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The Role of Early Experience in the Development of Acoustic Mating Behaviors of *Physalaemus pustulosus*

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The Role of Early Experience in the Development of Acoustic Mating Behaviors of *Physalaemus pustulosus*

by

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Dedication

For Taylor

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The Role of Early Experience in the Development of Acoustic Mating Behaviors of *Physalaemus pustulosus*

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Acoustic signals are used for communication in a variety of social contexts. Signals and receiver responses can vary, and that variation can come from development processes, physiological factors, or cognitive processes such as learning. The process of learning encompasses social learning, sexual imprinting, and vocal learning, among others. The development of acoustic behaviors has not been studied in anurans, one of the model systems for acoustic communications. This study examines the contribution of early experience in the development of the acoustically linked mating behaviors in *Physalaemus pustulosus*. I reared frogs from tadpole stage through metamorphosis to sexual maturity in four treatments – conspecific chorus, heterospecific (*P. enesefae)* chorus, isolation, and noise. I then measured the advertisement calls of males, mate choices of females, and vocal responses of males. The male calls differed slightly with early experience; males who were reared in isolation produced shorter calls that were less attractive to females than species typical calls. In phonotaxis tests, female mate choices showed no effect of early experience. The vocal responses of males to acoustic stimuli

showed the largest effect of experience. Males who were reared hearing a chorus of congeners, *P. enesefae,* increased their overall rate of calling and the production of complex calls when presented with a *P. enesefae* stimulus. By contrast, in the same test, males from other rearing groups showed either little increase or a decrease in calling activity. These findings support the prediction that female behaviors are less subject to environmental influence than are male behaviors. This research suggests that environment may play a role in the variation of male anuran behaviors and highlights the need for more research on the interaction of genes and environment in the development and variation of anuran mating behaviors.

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Chapter 1: Introduction

> *Animals talk to each other, of course. There can be no question about that … some animals have only a limited education and use very simple words and scarcely ever a comparison or a flowery figure; whereas, certain other animals have a large vocabulary, a fine command of language and a ready and fluent delivery* … (Twain, 1880)

Animal acoustic communication, the transmission of a signal from a signaler to a receiver, is used in a wide variety of social contexts (Bradbury & Vehrencamp, 1998). For example, bats use acoustic signals to identify group mates that are familiar to them within the context of the larger colony in which they reside, helping to establish social bonds among conspecifics (Boughman & Wilkinson, 1998). Vervet monkeys produce loud alarm calls that turn their group's attention to predators (Seyfarth et al., 1980). In a mating context, male frogs and toads (anurans) use acoustic advertisement calls to attract mates (Blair, 1941).

There can be advantages of variation, to both signaller and receivers. Vervet monkeys use minor variations in their alarm call to distinguish between avian and terrestrial predators (Seyfarth et al., 1980). Female anurans use individual variation in male advertisement calls as the basis for mate choice (Wells, 1988). Inappropriate or unusually large variations in calls can lead to errors in communication, which is especially important when sympatric congeners rely on similar signals and receiver responses for social behaviors, such as mating. To the extent that acoustic communication plays a role in mating and survival, as is the case with advertisement and alarm calls, variation in calls can contribute to reproductive isolation and speciation.

Variation in mating calls can be caused by anatomical changes with age, seasonal hormonal changes, and forms of learning such as observational learning or sexual

imprinting. Research on the contribution and interaction of innate factors and experience in the development of acoustic communication systems can identify potential sources of variation and the role of this variation in speciation.

LEARNING

Learning is "a change in state resulting from experience" (Shettleworth, 1998) or more descriptively, "adaptive and relatively permanent change in individual behavior as a result of past experience" (Slater, 1999). This change must result from individual or observational experience, not from physiological processes such as hunger. Forms of learning include conditioning, imprinting, social or observational learning, and vocal learning (Shettleworth, 1994; Domjan, 2003).

Examples of all of these forms of learning are found in a wide variety of taxa. Pavlov famously demonstrated conditioning in an experiment that paired a stimulus (bell) with a reward (food) to elicit a consistent response (salivation) in dogs (Pavlov, 1927). Imprinting was first demonstrated in precocial birds, which develop a preference for the first thing they see after hatching (Lorenz, 1935/1970) a type of learning now known as filial imprinting. By comparison, sexual imprinting involves the contribution of experience to adult mating preferences. Most cases of learning involve some type of social interaction. Relationships among kin or conspecifics often provide the opportunities required for learning, and communication systems offer a critical context in which learning takes place.

VOCAL LEARNING

Vocal learning is the acquisition of acoustic signals from early social interactions (Boughman & Moss, 2003) and is a form of observational learning that incorporates experience with others, as well as providing sensorimotor practice. Although other

authors differ somewhat in the words they choose to define vocal learning, most emphasize the importance of social interactions and the use of those vocalizations in species-specific signaling. Understanding the role these behaviors play in evolution begins with research on the mechanisms of vocal learning, the context in which it occurs, and the variation possible in both signalers and receivers.

Vocal learning is relatively rare, but has been observed in a wide range of animals. The most famous examples of vocal learning occur in the oscine passerines, the songbirds, but not all oscine passerines learn their songs (Nottebohm & Nottebohm, 1971). Two other groups of birds exhibit vocal learning, including non-passerine hummingbirds (Baptista & Schuchmann, 1990) and parrots (Hall et al., 1997). There is some correlative evidence that bellbirds, a suboscine passerine, also learn their song (Saranathan et al., 2007). Bats are generally considered vocal learners (Esser, 1994). Some marine mammals show behaviors related to vocal learning. Dolphins are capable of learning vocal signals (Richards et al., 1984; Fripp et al., 2005; Tyack, 2001). Harbor seals can mimic vocal signals (Ralls et al., 1985), and changes in the social songs of humpback whales may be indicative of vocal learning (Payne & Payne, 1985). More recently, evidence suggests elephants may use vocal learning to acquire their social signals (Poole et al., 2005).

The role of vocal learning in humans is generally accepted, but it has only been studied in limited instances with deaf children (Lenneberg, 1967), or on very rare occasions with socially isolated children (Curtiss, 1977). Research on non-human primates has yielded confusing, and in some cases, directly contradictory results (summarized in Egnor & Hauser, 2004). In experiments on isolation-reared macaques (Newman & Symmes, 1974), the authors confound acoustic isolation with social isolation and attribute abnormal vocalizations to the lack of a conspecific tutor, when the isolation

from individuals could have produced cognitive deficits, especially in such a social species. This finding, in particular, has not been corroborated. Other research describes changes in vocal responses of adults, but not ontogenetic changes in juveniles. In chimpanzees, the acoustic differences between tribes of wild chimpanzees are correlated with geographic distance, but not genetic relatedness or habitat structure (Crockford et al., 2004). These findings may demonstrate that genes or habitats do not contribute to variation in vocal signals in these species, but, at the same time, they cannot be taken as positive evidence of vocal learning.

Vocal learning is demonstrated most convincingly in controlled experiments that compare the songs of animals reared with conspecific song to those raised in relative acoustic isolation or by heterospecific tutors. Pointing out that it is logistically difficult to perform those experiments with most of the non-avian taxa for which vocal learning has been posited, is an understatement.

Songbirds are the classic model system for vocal learning. The first issue of the prestigious avian research journal, *The Auk,* included an article on bird song in which the author comments that no one has thought to study "the singing of our birds" scientifically (Bicknell, 1884). He hints that there might be "stimulating investigation" possible in this field. In the early mid-1900s, the development of microphones and tape recording equipment by Bell Laboratories and affordable analysis equipment by Hewlett-Packard and others stimulated such investigations and there was a subsequent increase in the scientific publications on bird song (Thorpe, 1961).

Thorpe led the field with his comprehensive studies on the function of bird song; his lab experiments showed that chaffinches reared in isolation produced atypical songs (Thorpe, 1958). Soon after, observations of dialects in populations of white-crowned sparrows in central and northern California (Marler & Tamura, 1962) led to experimental

studies of lab-reared sparrows, which showed that they acquire their songs by cultural transmission (Marler & Tamura, 1964).

Most subsequent studies focused on song acquisition by males. Male sparrows reared without exposure to conspecific tutors produced songs that were significantly different from their species' typical song (Marler, 1970). This exposure to conspecific tutors must occur during a sensitive period (Marler, 1984), but new evidence shows that this sensitive period can extend into adulthood (Woolley, 2004). Sparrows reared in acoustic isolation, with no exposure to bird song, use the auditory feedback from their own song to learn some aspects of their species' song; deafened birds produce songs that are typical of the species in some respects, but otherwise atypical and unstable (Konishi, 1965). This work suggests that some aspects of song are innate, and not learned, and that there are substantial differences in the degree to which learning leads to the production of typical song by adults. For example, migratory species acquire their song more quickly than non-migratory congeners (Nelson et al., 1995).

The diversity in songbird behavior led researchers to study the brain of songbirds, where the neural circuitry was first identified (Nottebohm & Arnold, 1976). Neurons in key regions of the brain grow in response to auditory stimuli and die without that stimulation (Konishi & Akutagawa, 1985). An anatomical substrate was also shown for the sensitive period (Konishi & Akutagawa, 1988). Beginning in the early 1990s, researchers began to focus on the expression of immediate early genes in key regions of the brain in response to hearing and producing song (Mello et al., 1992).

Research on the production of birdsong typically focuses on males, but in some species, both sexes produce songs. In cardinals, males and females learn and produce sexually dimorphic songs (Yamaguchi, 1998a) that are different enough to be used to identify the sex of the singer (Yamaguchi, 1998b). The mechanisms whereby females

learn songs may be different from males, as well. Females begin learning songs at approximately the same age as males, but they have a shorter sensitive period; isolation-reared females are not able to improvise species typical songs, whereas males do (Yamaguchi, 2001). Female song may not be just an oddity; one group of researchers suggested that the female song might be the ancestral condition for oscine passerines (Riebel et al., 2005). Oscine passerines probably evolved in Australasia and most examples of female song are observed in extant Australian species.

SEXUAL IMPRINTING

Sexual imprinting is another form of learning in which individuals acquire mate preferences based on early experience (Lorenz, 1935/1970). Few studies have considered the mechanisms underlying the acquisition of preferences by female birds for male song, an oversight that has not gone unnoticed (Riebel et al., 2005). It is an oversight because song dialects are thought to contribute to reproductive isolation, but isolation would only be complete if song preferences also vary with experience. Zebra finches prefer the familiar call of their father even though their exposure to that song was limited to juvenile experience (Miller, 1979). Juveniles raised with tutors showed preferences as adults for that song, whether it was the song of their own species or the substantially different song of another subspecies (Clayton, 1990). Male and female zebra finches both preferred conspecific song even when reared in acoustic isolation (Braaten & Reynolds, 1999), suggesting that mate preferences, like male song itself, may arise from innate templates that are shaped by experience.

Sexual imprinting occurs in other taxa as well, including invertebrates. Female wolf spiders prefer the phenotype that they were exposed to as juveniles (Hebets, 2003). Subadult spiders were reared with exposure to either the natural pattern of forelegs or an artificially colored pattern of forelegs. When adults, females chose the phenotype with

which they were reared, demonstrating that the mechanisms that facilitate sexual imprinting may be present in a wide range of taxa.

MALE-MALE COMMUNICATION

Learning can influence male song and female responses, but it also can affect the response of other males. Male birdsong also serves to establish territories with other males (summarized in Catchpole & Slater, 1995; Marler & Slabbekoorn, 2004). Males compete for resources and territories. Acoustic signals can evolve to become proxies for physical prowess, which allows males to avoid risky physical competition (Fisher, 1930). For example, the number of rattles produced by male barn swallows increases when other males approach (Galeotti et al., 1997). If the signal is honest, then it should correlate with actual fighting condition, such as size or aggression. In barn swallows, increased song rate is correlated with higher testosterone levels, larger body size, and better overall condition.

Some species' songs can contain elements that both attract females and threaten males. In playback experiments with warblers, one syllable of the song caused other males to concede territory (Jarvi et al., 1980). Across a wide range of species, playing the call of a male on a speaker decreases the likelihood that other males approach that area (Marler & Slabbekoorn, 2004). By contrast, playing male birdsong increases the visitation rate to those sites by conspecific females (Eriksson & Wallin, 1986).

Compared with the research on the contribution of learning to male song production and female responses to song, research on the role of learning in male-male interactions is in its infancy. Recent studies focus on the function of song in male-male interactions (see above), but there has been little research on how males acquire acoustic behaviors for intraspecific competition. However, one study showed that male white-crowned sparrows respond more vigorously to a local dialect then a foreign one (Nelson

& Soha, 2004). However, whether early experience contributes to this differential response remains unknown.

NATURE VS. NURTURE

> *The epigenesis of behavior is guided by a complex mix of innate and experiential factors* (Lindsay, 2000).

Much of the research on the development of song in oscine passerines took place during the nature vs. nurture debate of the mid-twentieth century. Behaviorists presented evidence such as filial imprinting in birds that demonstrated the overwhelming role of nature (Lorenz, 1937). Others argued passionately that the interaction of heredity and environment is formative in the development of behaviors (Hebb, 1953; Lehrman, 1953). Tinbergen (1963) called for a more balanced approach with an emphasis on understanding the causal mechanisms of behavior, irrespective of whether the behavior is innate.

Recent research reveals a more complex reality and the false dichotomy between nature and nurture, with more researchers now investigating the subtle interactions between genes and environment. Studies on hormones to maternal care, in such diverse taxa as voles and flies, show that experience and genetics often interact in complex ways (Robinson, 2004).

Research on songbirds has uncovered specific gene by environment interactions. Deafening or isolating young male finches leads to the inhibition of key enzymes that play a role in the synaptic plasticity critical for song learning during the sensitive period (Sakaguchi & Yamaguchi, 1997). Male and female zebra finches show an early behavioral bias toward conspecific song, suggesting that there is an innate mechanism with which vocal learning interacts (Braaten & Reynolds, 1999). Another model system for vocal learning, humans, has provided the most recent breakthrough. The FoxP2 gene

has been implicated in human families with developmental problems that affect the acquisition of language (Lai et al., 2001). This gene is differentially expressed in vocal songbird learners (Haesler et al., 2004), leading to increased speculation that it plays a key role in the gene by environment interactions that lead to the acquisition of language in vocal learners (Scharff & White, 2004). However, FoxP2 is not under selection in other presumed vocal learning mammals, such as whales and bats (Webb & Zhang, 2005).

ANURAN COMMUNICATION

One of the original researchers in birdsong commented that taxa from insects to amphibians to mammals have elaborate vocalizations that might be of interest (Thorpe, 1961). Our understanding of animal communication has benefited from research into the acoustic behaviors of anurans (reviewed in Gerhardt & Huber, 2002; Ryan, 2001). Anuran mating systems are linked to three acoustic behaviors: male advertisement calls, female preferences for particular types of calls, and the male response to the calls of nearby males.

Advertisement calls produced by males have long been shown to be a potential reproductive isolating mechanism (Blair, 1941). This mechanism was reinforced when it was demonstrated that females are attracted to conspecific calls (Martof & Thompson, 1958) and that they can discriminate between closely related species solely on the basis of advertisement calls (Littlejohn & Michaud, 1959). For many anurans, the behavioral variation among and between species, the physiological and anatomical structures, and the endocrine and neural systems have been studied extensively (Ryan, 2001).

Female anurans not only use calls to recognize conspecifics, but also often rely on calls to express mate preferences. These preferences are typically studied using a two-choice test where females are placed in an appropriate enclosure and presented with two

acoustic stimuli. The experimental design relies on the choice of stimuli. Recognition of conspecifics is tested by determining if a single call elicits phonotaxis, typically by presenting females with the call of their own species and that of another species.

Males are also receivers in this mate communication system. Males often exhibit vocal responses to advertisement calls in a form of reciprocal exchange that can also lead to a change in the signals (Bradbury & Vehrencamp, 1998). In some species, this exchange can help establish physical territories (Lutz, 1960). Male bullfrogs and cricket frogs lower the dominant frequency of their call, possibly attempting to appear larger in size, when they detect nearby conspecific males calling (Bee & Bowling, 2001) (Wagner, 1992). White-lipped frogs adjust the dominant frequency of their call to match that of a played stimulus (Lopez et al., 1988).

The anatomy and physiology of anuran audition have been studied in depth, which has led to much insight on the causation and function of acoustic signals in anuran behavior. Frogs have two primary auditory papillae: the amphibian papilla (AP) and the basilar papilla (BP) (see Wever, 1985). The AP responds to lower frequency sounds and the BP to higher frequencies (Zakon & Wilczynski, 1988). The observation that calls and hearing were correlated led to the hypothesis that the frequency response of the AP end organs should match the frequencies present in conspecific advertisement calls (Capranica, 1965). This “matched filter hypothesis” is well supported by empirical data (Gerhardt & Schwartz, 2001). The neural pathway of anuran audition runs from the periphery, through the midbrain, and then projects to areas of the forebrain implicated in the initiation of behavior (Feng & Schellart, 1999). Mapping the functional regions of the auditory midbrain showed that complex processing occurs simultaneously in multiple areas (Hoke et al., 2004). In both sexes of frogs, hormone physiology plays a critical role in receptivity of individuals to mating behaviors. Arginine vasotocin, a drug that

increases gonadal hormone production, increases the likelihood of calling in male anurans. Gonadal steroids, in turn, can elicit receptivity in females (Emerson & Boyd, 1999). Hearing advertisement calls increases the number of gonadotropin-releasing hormone immunoreceptive cells in male tree frogs (Burmeister & Wilczynski, 2005), providing evidence for a link between anuran acoustic signals and changes in peripheral neurophysiology.

TÚNGARA FROG

The focus on the *Physalaemus pustulosus* species group of Neotropical frogs began with the observation that male túngara frogs *(P. pustulosus)* are capable of producing a simple (whine) or complex (whine-chuck) advertisement call. The whine is an amplitude-modulated downward frequency sweep and the chuck is a short high amplitude call with energy across a broader range of frequencies. The escalation from simple to complex calls is facultative and females prefer the complex call (Rand & Ryan, 1981; Ryan, 1980). Frog-eating fringe-lipped bats preferentially prey on túngara frogs making complex calls (Tuttle et al., 1982; Ryan et al., 1982).

Female túngara frogs recognize their own species call and discriminate between conspecific males. Offering the choice of a whine or a whine-chuck in a standard phonotaxis experiment tests a female's responsiveness to mate. Females who are responsive to mating will choose one or the other; female túngara frogs overwhelmingly choose the complex whine-chuck. Females recognize their own species call over that of heterospecifics, both allopatric and sympatric (Ryan & Rand, 1993).

Males typically choose to make the complex call only when other males are also calling (Ryan, 1985). They also escalate to the complex call when presented with heterospecific calls to which suffixes have been appended (Ryan & Rand, 1998). Combined with female preferences, this escalation shows that both male and female

túngara frogs have a pre-existing bias for a stimulus that is more elaborate than the conspecific advertisement call.

The genus *Physalaemus* includes species distributed from Middle America through northern South America to the Pacific Coast west of the Andes. Most species are allopatric with *P. pustulosus* with the exception of *P. enesefae*, which is sympatric to *P. pustulosus* in Venezuela (La Marca, 1992). To avoid the high cost of breeding with heterospecifics, females in the area of sympatry must discriminate against the calls of *P. enesefae* and they do so in a probabilistic fashion (Ryan et al., 2003). Researchers synthesized a series of calls intermediate between *P. pustulosus* and *P. enesefae* and demonstrated that female túngara frogs responded to calls all along this continuum. Their results showed that female permissiveness tapers off as acoustic distance increases. Synthetic calls created in this study could be used to test permissiveness toward non-species calls in female túngara frogs.

THE ROLE OF EXPERIENCE

Despite wide-ranging research on anuran communication, the possibility that learning plays a role in the development of these behaviors has received little attention. Tadpoles are capable of learning kin discrimination possibly through imprinting on olfactory cues (Hepper & Waldman, 1992; Waldman, 1987). Other experiments, such as one in which anurans were conditioned to perform behavioral audiograms (Megela-Simmons et al., 1985), demonstrate the capacity of anurans for operant conditioning with non-ecologically relevant tasks, but do not address the degree to which learning takes places in more natural contexts.

Does learning contribute to the development of acoustically linked mating behaviors in anurans? Given the importance of communication in frogs, and their

acoustically linked mating system, it is perhaps surprising that there are no published studies of the role of early experience in the acquisition of acoustic behaviors.

If experience with conspecific tutors were a requirement for the development of species-typical advertisement calls in adult male túngara frogs, then this species would be classified as a vocal learner, along with songbirds, marine mammals, and other taxa. Another possibility is that experience modulates or somehow alters the development of calls in túngara frogs. Another possibility is that experience does not produce changes in adult male vocalizations. Such a finding would validate the long-held assumption by anuran researchers that experience does not contribute to variation in adult advertisement calls.

It is possible that experience alters female mate preferences, suggesting that sexual imprinting plays a role in the acquisition of mating preferences, as demonstrated in phonotaxis experiments that test recognition and discrimination. Sexual selection theory suggests that female preferences are more likely invariable when male-female behaviors are coevolved; results should be analyzed in the context of male and female choices.

The role of learning can be studied in male-male interactions, using experiments that test evoked vocal responses. The role of learning in male-male competition is less understood, even in species in which vocal learning has been demonstrated. Perhaps the bias comes from the assumption that male-male behaviors are more variable, and play a less significant role overall in sexual selection and mating success, than male calls and female preference.

If frogs learn acoustic behaviors, then there are developmental and evolutionary consequences. Learning provides a mechanism for continuous change and adaptation. The behaviors of animals that attend to conspecific tutors, whether for vocal learning or sexual imprinting, might be influenced by the arrival of new species. When conspecific

tutoring is a strict requirement, dialects typically develop as small population-level differences arise in geographically separated groups. Shifting female preferences could change the selective force on males. By contrast, males that require early experience to develop appropriate vocal responses to conspecifics might suffer from reduced mating opportunities. Even a discovery of sexual imprinting might only show that anurans have some cognitive precursors to vocal learning.

On the other hand, if anuran acoustic behaviors are not influenced by environment and experience, then their vocal communications must be the product of innate mechanisms, perhaps determined exclusively by genetic factors that do not interact with environmental factors. If this is true, then it confirms that anurans are a good study system for identifying the genes, as well as the physiology and anatomy, underlying mating behaviors.

One argument against vocal learning in anuran communication is the lack of true geographic dialects in anurans. Geographic variation in mating calls has been documented in many species (Asquith et al., 1988; Nevo & Capranica, 1985; Capranica et al., 1973; Ryan & Wilczynski, 1991; Ryan et al., 1996); however, in no case was there evidence for the sharp boundaries that are associated with songbird dialects. The lack of dialects in anuran mating calls, however, does not preclude the possibility that early experience influences acoustically linked mating behaviors.

Another argument against vocal learning in anurans is that the tadpoles of some anurans metamorphose after the adults have left the breeding site. In these species, juvenile frogs attain sexual maturity without experiencing adult mating behaviors but this is not the case in all temperate zone species, nor is it the norm for tropical anurans. In some anurans, including *P. pustulosus,* metamorphosis and adult mating behaviors overlap in time and space.

Learning is not universal across taxa or across behaviors, in part because the benefits of it and the factors that encourage evolution of learning are often balanced by costs. Changes in environmental factors can influence the likelihood that learning will evolve (Stephens, 1991). The learning is more likely to occur when consistent conditions during an organism's lifetime increase the value of learning but changes across generations increase the payoff of learning. In the specific context of mating, learning is advantageous for males when their encounter rate with receptive females is high (Dukas et al., 2006). Learning is by no means a cost-free endeavor. Selection for learning has been found to be accompanied by decreased competitive ability of larvae in fruit flies (Mery & Kawecki, 2003). Male anurans do experience consistent environments during mating and high encounter rates with receptive females, perhaps suggesting that selection may favor learning at least in males.

Learning also does not have to be universal across sexes. Differential parental investment predicts that female behaviors will be innate and invariant while selection favors males that exhibit relatively more variation in their behaviors (Trivers, 1972). Female anurans may exercise mate choice only once in a lifetime while males are often presented with numerous mating opportunities, especially in species like *P. pustulosus* that are prolonged breeders. Parental investment predicts that males may be permissive in terms of the contribution of early experience to the development of their behaviors, while females may be much less so.

To date, there is no evidence that early experience plays any role in the development of acoustically linked mating behaviors in anurans. In fact, no studies have been published that address the role of early experience in acoustically linked mating behaviors in anurans. One of the earliest major works on the subject of amphibians discusses the function of mating behaviors and the developmental physiology of

amphibians, but makes no mention of developmental processes that might contribute to the acquisition of these behaviors (Dickerson, 1906). Subsequent reviews of amphibian biology and anuran communication published over the past 100 years address the causation, function, and evolution of mating behaviors but do not discuss or even mention the question of developmental processes underlying these behaviors (Noble, 1931/1954; Duellman & Trueb, 1986; Gerhardt & Huber, 2002). Without such understanding, the alternative hypothesis that most variation in such behaviors is determined by genetic mechanisms alone cannot be accepted. Given the importance of anuran communications in studies of brain, behavior, and evolution, this hypothesis should be tested empirically.

The experimental design used in birdsong studies could inform research on the potential contribution of learning to the acquisition and development of acoustically linked mating behaviors in anurans. This study evaluates mating behaviors in male and female *Physalaemus pustulosus* to better understand the extent to which early experience contributes to the acquisition of male calls, female preferences, and male evoked vocal responses.

Chapter 2: Early experience leads to changes in the advertisement calls of male *Physalaemus pustulosus*

SUMMARY

Anurans have long been a model system for studies of acoustic communication but little is known about how their acoustically-linked mating behaviors develop. In this study, we reared *Physalaemus pustulosus* in four acoustic treatment groups: 1) hearing a conspecific chorus of *P. pustulosus,* 2) acoustically isolated from all frog calls, 3) hearing a chorus of the congener *P. enesefae,* and 4) hearing noise. We then measured the spectral and temporal characteristics of the calls produced by males and tested them for differences between treatment groups. The isolation-reared males produced calls that were shorter in duration. Females showed discrimination against these shorter calls. Early experience alters the advertisement call of male *P. pustulosus* and acoustic isolation alters the calls in a way that decreases their attractiveness to females. Male calls may be the product of a gene by environment interaction.

INTRODUCTION

Vocal learning is the acquisition of acoustic signals from early social interactions and has been extensively studied in songbirds. Observations of dialects in populations of white-crowned sparrows in central and northern California (Marler & Tamura, 1962) led to studies of lab-reared sparrows, which showed that they acquire their songs by cultural transmission (Marler & Tamura, 1964). Atypical songs are produced when males are reared in isolation (Thorpe, 1958) or with heterospecific tutors (Marler & Tamura, 1964). Tutoring must occur during a sensitive period (Marler, 1984); however, more recent studies show that this period extends into adulthood (Woolley, 2004). Divergence in

birdsong may be a reproductive isolating mechanism in speciation (Seddon & Tobias, 2007).

Another classic model for studies in animal communication is the acoustic behavior of anurans (reviewed in Gerhardt & Huber, 2002; Ryan, 2001). Advertisement calls produced by male frogs and toads have shown to be a potential reproductive isolating mechanism (Blair, 1941). This was reinforced when it was demonstrated that females are attracted to conspecific calls (Martof & Thompson, 1958) and that they can discriminate between closely related species solely on the basis of advertisement calls (Littlejohn & Michaud, 1959).

One of the original researchers in birdsong commented that a wide range of taxa – from insects to amphibians – have elaborate vocalizations that might be of interest in studies of vocal learning (Thorpe, 1961). Geographic variation in mating calls has been documented in many anurans (Asquith et al., 1988; Nevo & Capranica, 1985; Capranica et al., 1973; Ryan & Wilczynski, 1991; Ryan et al., 1996); however, in no case was there evidence for the sharp boundaries that are associated with songbird dialects. The lack of dialects, however, does not preclude the possibility that early experience influences anuran mating behaviors.

Given the importance of communication in anuran reproductive behavior, it is surprising that there are no published studies about the role of early experience in the acquisition of anuran acoustic behaviors. If experience with conspecific tutors were a requirement for the development of species-typical advertisement calls, then anurans would be classified as vocal learners, along with songbirds, marine mammals, and some other taxa. Another possibility is that early experience modulates or somehow alters the development of anuran calls, perhaps because of gene by environment interactions, suggesting that although their calls may depend largely on innate processes, some critical

experience is also required. Finally, it is possible that there is strong to absolute control of genes on call development with little detectable effect of the environment.

If frogs learn acoustic behaviors, then there are developmental and evolutionary consequences. Learning provides a mechanism for continuous change and adaptation. The behavior of animals that attend to conspecific tutors might be influenced by the arrival of new species. When conspecific tutoring is a strict requirement, dialects typically develop as small population-level differences arise in geographically separated groups.

In a well-studied Neotropical frog, *Physalaemus pustulosus,* males produce either a simple (whine) or complex (whine-chuck) advertisement call (Figures 1 and 2). The whine is an amplitude-modulated, downward frequency-sweep. The escalation from simple to complex calls is facultative and females prefer the complex call (Rand & Ryan, 1981; Ryan, 1980). Males typically choose to escalate to the complex call only when other nearby males are calling (Ryan, 1985). Frog-eating bats preferentially prey on males making the complex call (Tuttle et al., 1982; Ryan et al., 1982).

The genus *Physalaemus* is widespread throughout South America, with *P. pustulosus* being the sole member of the species in Middle America. *P. pustulosus* is allopatric with most species with the exception of *P. enesefae,* which is sympatric in the llanos of Venezuela (La Marca, 1992). Male *P. enesefae* produce only simple calls, with a similar downward frequency sweep, but their calls are longer in duration compared to those of *P. pustulosus* (Figure 3). To avoid the high cost of breeding with heterospecifics, females in the area of sympatry must discriminate against the calls of *P. enesefae* and they do so in a probabilistic fashion (Ryan et al., 2003). In addition, female *P. pustulosus* discriminate against a number of synthetic calls that are intermediate between the conspecific call and the *P. enesefae* call. Their results show the point where female

response reaches a specific level, so those synthetic calls can be used to test thresholds for female preferences.

In this study, we use the classic experimental design used in songbird research to investigate the possible contribution of early experience to the development of advertisement calls in male *P. pustulosus*. Acoustic rearing conditions provide a range of early experiences against which we test the effects of this experience on the advertisement call of male túngara frogs.

EXPERIMENT ONE: METHODS

Three hundred *P. pustulosus* from 20 broods were reared from tadpoles through sexual maturity in sound-attenuated enclosures approximately 32 cm wide, 60 cm long, and 18 cm tall. Each brood was separated into one of four rearing conditions: 1) *P. pustulosus* chorus, 2) isolated from all frog calls, 3) *P. enesefae* chorus, and 4) noise. The playback for groups 1 and 3 were broadcast at 80 dB SPL, approximately the amplitude of a calling male measured at 1 meter, and played for 12 hours during each night photoperiod. The noise was also broadcast at 80 dB SPL, but played for 24 hours. All animals were fed ad libitum and received 12 hours of broad-spectrum light daily. No standing water was present in the enclosures; this species needs water to call, so the lack of standing water prevented males in the enclosures from calling. Four enclosures received each rearing treatment (replicates), for a total of 16 enclosures. All frogs were maintained in these acoustic environments for 8 to 10 months, until they reached sexual maturity (24 mm snout-vent length).

At sexual maturity, males were placed in individual sound-attenuated chambers (22 cm wide, 24 cm long, 40 cm tall) and presented with small ponds of water from which to call. A chorus of *P. pustulosus* was played for 30 minutes using Altec-Lansing VS2320 speakers placed in each chamber and connected to a Dell 2350 computer that

played the sound files. Vocalizations made by the focal male were recorded on a Juster MP-018 microphone attached to a Dell 2320 computer running the Linux operating system. The sox sound utility software program was used to save the input from the microphones to digital sound files; vocalizations produced by the focal male were identified manually using the CoolEdit (Syntrillium) software program. Males who did not call the first night were retried for as many as five consecutive nights before being disqualified; most males called during the first or second attempt. Thirty males from each rearing group were successfully recorded. Thirty calls for each male from the beginning, middle, and end of his calling activity were analyzed for temporal and spectral characteristics using the Signal (Engineering Design) sound analysis environment. The values for the temporal and spectral characteristics for the 30 calls were then averaged to produce a single value for each characteristic for each male (Figure 2). For each call, the overall duration was measured in milliseconds. The rise time describes the time from the beginning of the call to the point at which the call reaches maximum amplitude and the fall time is the time from maximum amplitude to the end of the call. The initial frequency is the frequency at the beginning of the call and the final frequency describes the frequency at the end of the call, based on overall call duration. The data were evaluated first with a multivariate analysis of variance to test for effect of replicate (SPSS 15.0). The data were then analyzed using multivariate analysis of variance using a Tukey's post hoc test for pairwise comparisons.

EXPERIMENT ONE: RESULTS

Overall, there was a main effect of early experience treatment ($F=1.814$, $df=15$, $P=0.031$; Tables 1 and 2).

The duration of the calls among rearing groups was significantly different ($F=5.817$, $df=3$, $P=0.001$; Figure 4). Males reared in isolation produced calls that were

shorter in overall duration than the calls produced by males who were reared hearing the *P. pustulosus* chorus (P=0.016), the *P. enesefae* chorus (P=0.002), and noise (P=0.005). Overall call duration consists of the rise time, the time it takes for the call to reach maximum amplitude, and fall time, the time from maximum amplitude to the end of the call. The rise times were not significantly different across the rearing groups (F=0.523; df=3, P=0.667; Figure 5). The fall times of the calls were different among rearing groups (F=4.238, df=3, P=0.007; Figure 6). The fall times of the calls of males reared in isolation were shorter than those of males reared hearing the *P. enesefae* chorus (P=0.018) and the noise (P=0.011) and close to significantly different than the calls of males reared hearing the conspecific *P. pustulosus* chorus (P=0.081).

The initial frequencies of calls were slightly different among rearing groups but this difference was not significant (F=1.961, df=3, P=0.124; Figure 7). The final frequency of the calls produced by males in the four rearing groups was not significantly different (F=1.356, df=3, P=0.260; Figure 8). The initial frequency of the calls of males in all treatment groups was lower than the initial frequency of calls of males recorded in Panama (Ryan & Rand, 2003b). The duration and fall times of the calls presented here are shorter than the duration and fall times reported in that study.

EXPERIMENT TWO: METHODS

Based on the finding that the isolation-reared males produced calls that were significantly shorter in duration and fall times, female *P. pustulosus* from the isolation-rearing group and the *P. enesefae* rearing group were tested for their discrimination of calls with longer or shorter duration times. *P. pustulosus* calls were synthesized using the mean call characteristics of the males reared hearing the *P. enesefae* chorus. These calls were approximately 50 ms longer in duration than calls produced by naïve-reared males. To test the threshold for discrimination, calls that were 25 ms longer and 10 ms longer

were also synthesized. These calls were paired with synthetic calls with the same duration as the calls produced by the isolation-reared males. The calls of the males from the *P. enesefae* reared group were not significantly different in duration from the calls of the males in the *P. pustulosus* and noise rearing groups.

Twenty-eight adult females reared in isolation and reared hearing *P. enesefae* (described above) were injected with 250 IU of human chorionic growth hormone to simulate the hormonal state of wild females found in amplexus (Lynch et al., 2006). These females were tested in a 2.7 x 1.8 meter acoustic chamber equipped with an infrared camera for observation. Calls were presented antiphonally from two speakers placed at the long ends of the chamber. Each call was repeated every two seconds, which is the typical call rate for two calling male *P. pustulosus,* and played at a sound level of 81 dB SPL (re: 20 μmPa). Each female was placed in the center of the chamber and held under a funnel for two minutes while the stimuli were presented, then allowed to move freely. Choices were scored when the female 1) moved from the center within five minutes, 2) continued to move at least every two minutes, or 3) came within 10 cm of one speaker within fifteen minutes of being released. Each female was initially tested for their motivation to mate using conspecific simple and complex calls. If a female did not make a choice in this test of conspecific calls, she was not tested further. Female choices for each group were compared to the null hypothesis with an exact binomial probability (SPSS 15.0).

EXPERIMENT TWO: RESULTS

More females were more likely to choose the call that was 50 ms longer in duration (P=0.023; Figure 9 and Table 3). Females showed no preference for calls that were 25 ms longer (P=0.999) or 10 ms longer (P=0.839).

DISCUSSION

These results show that males develop typical mating calls regardless of their early experience. Early experience does, however, alter details of the advertisement call of male *P. pustulosus* in a manner that is salient to female *P. pustulosus*. Lack of exposure to frog calls causes male túngara frogs to produce calls that are shorter in duration. Call duration is one of the factors used by females to choose a mate and shorter calls are less attractive to females, demonstrating that experience may contribute to relevant variation in the advertisement calls used in female mate choice in this species.

These results support the assumption that frogs do not require access to conspecific tutors to learn their calls. The large-scale deficits in acoustic signals observed in isolation-reared songbirds were not observed in any of the treatment groups in this experiment, and the calls of the males reared with heterospecific tutors were no different than those of the males reared hearing their own species calls. Lack of experience alters the calls of these males, but there is no evidence for vocal learning in this anuran species.

The temporal and spectral features of the calls of the males studied differ from those of males studied in Panama (Ryan & Rand, 2003b). This may be due to housing conditions in the laboratory or conditions during call recordings. Factors such as body size, temperature, humidity, and reproductive status may affect the calls of this species. Calls recorded in the wild are typically of males active at the ponds during mating season where the calls recorded in this study were obtained from naïve males. The question of how early experience affects male calling that we address in this study is one that requires the use of lab-reared animals. The differences between calls produced by lab-reared males and those in the wild may require further study.

There was a treatment effect on the duration of the calls. The change may not be the result of any specific experience, but of the consequences of insufficient acoustic

stimulation. The typical calls made by the heterospecific-tutored males and the noise-reared males suggest that a specific structure of the acoustic stimuli is not necessary to elicit normal call development in túngara frogs. Instead, there may be a threshold of minimum acoustic stimulation necessary for the auditory peripheral and central neural processes to develop. Although these frogs were not raised in absolute silence, they had an overall lower level of acoustic stimulation than the frogs in the other three rearing groups. In starlings reared with heterospecific tutors, males still developed stereotypical conspecific song (Boehner & Todt, 1996). Similarly, acoustic stimulation in túngara frogs may trigger an innate mechanism by which normal song then develops. In this case, the *P. pustulosus, P. enesefae,* and noise-reared groups met some threshold of stimulation, whereas the acoustically isolated males, although not kept in absolute silence, had overall less acoustic input. Song deprivation affects the expression of an enzyme critical to proper development of the neural circuitry in zebra finches (Sakaguchi & Yamaguchi, 1997). Acoustic stimulation interacts with gonadal hormones to produce normal vocal centers in canaries (Bottjer et al., 2004). To better understand if similar mechanisms are operating in frogs, future studies should evaluate the hormone and neurological changes in acoustically deprived anurans.

The effects of acoustic deprivation on male calls, whatever the mechanism, appear to be sufficient to influence female mating call preferences. Females reared in the same four acoustic treatment groups used in this study, consistently preferred the longer calls produced by males that were not acoustically isolated. This discrimination against the shorter calls produced by acoustically isolated males could lead to reduced reproductive success in those males.

These data provide empirical support that anurans do not learn how to produce species-typical advertisement calls. Instead, it may be that the development of anuran calls is controlled by genetic factors that are not influenced by early experience.

Chapter 3: Female preferences are not altered by early acoustic experience in the túngara frog, *Physalaemus pustulosus*

SUMMARY

In the two extremes female mate choice preferences may be acquired through experience or prescribed by innate genetic mechanisms. Understanding the relative contribution and possible interaction between these mechanisms is important for identifying sources of variation in mating behaviors. In this study, we evaluate the contribution of early experience on the acquisition of mate choice preferences in a Neotropical frog, the túngara frog, *Physalaemus pustulosus*. We reared frogs from tadpole stage through sexual maturity in one of four acoustic treatments: 1) hearing a conspecific chorus of *P. pustulosus,* 2) acoustically isolated from all frog sounds, 3) hearing a heterospecific chorus of the closely related sympatric congener *P. enesefae,* 4) hearing noise. At sexual maturity, we tested each female for her preferences for conspecific complex versus simple calls, her recognition for calls of *P. enesefae,* and her ability to detect noise-masked calls with varying levels of mask. Female choices in all of these tests were consistent with those seen in this species in previous studies on the wild. There was no effect of early experience on female preferences. In this first study of the acquisition of anuran female mating preferences, we conclude that *P. pustulosus* acquire their mate choice preferences solely through innate mechanisms.

INTRODUCTION

Animals can acquire behaviors through innate genetic processes, through experience, or through genes-by-environment interactions. Imprinting is one form of learning in which adult preferences are influenced by early experience (Lorenz,

1935/1970). Young birds acquire a preference for the phenotype of adults to which they are exposed as juveniles, a process known as filial imprinting.

Fewer studies examine sexual imprinting, the role of experience on the acquisition of mating preferences. In birds, it is known that female zebra finches preferentially recognize songs to which they were exposed as juveniles (Miller, 1979). Zebra finches appear to have pre-existing biases for conspecific song, however, because female finches reared in isolation prefer the conspecific song (Braaten & Reynolds, 1999; Clayton, 1988). These two studies in zebra finches highlight the need for good experimental design in studies of sexual imprinting (Riebel, 2003b). Adults should be reared in isolation and with heterospecific song to test for a predisposition for conspecific song. In addition, calls used for early experience should be different from those used for testing adults, to counter effects of familiarity on adult preferences. Studies of sexual imprinting in songbirds demonstrate that early experience can influence female preference but that the timing of this experience and its interaction with innate mechanisms is not understood (Riebel, 2003a).

The presence of this learning in other taxa is also poorly known. Early experience has been shown to affect female mate choice in invertebrates. Female wolf spiders were more likely to mate with males that exhibit the same secondary sexual traits of males to whom they were exposed during their last molt before reaching sexual maturity (Hebets, 2003).

Anurans are a classic model system for studies of acoustic communication. Male advertisement calls typically serve as the basis for female mate choice. Much is known about the causation, function, and evolution of male and female mating behaviors (Ryan, 2001; Gerhardt & Huber, 2002). Less is known about the acquisition of mating behavior and the relative roles of innate and experiential factors. The total lack of rigorous studies

on the role of early experience in the acquisition of anuran mating behaviors suggests an implicit assumption that early experience does not play a role in the ontogeny and acquisition of these behaviors. Given the importance of anuran mate recognition studies in the fields of species recognition and sexual selection this dearth of data is somewhat perplexing.

Here, we document the contribution of early experience to the development of female preferences for male mating calls in the túngara frog, *Physalaemus pustulosus,* a Neotropical frog found distributed in Middle and northern South America. . Males of this species produce either simple calls, whines, with no suffix, or complex calls that consist of a simple call followed by one or more suffixes, or chucks (Figure 1). Females typically prefer the complex call (Ryan, 1985). Females prefer conspecific calls to those of closely-related heterospecifics (Ryan & Rand, 1993). Conspecific call preferences are also exhibited versus calls that are synthetic intermediates between *P. pustulosus* and *P. enesefae* (Ryan et al., 2003).

The substantial background on acoustic communication of *P. pustulosus* makes that a useful system for investigating the role of early experience in the development of adult mating behaviors. In addition, these animals are easily raised and maintained in our breeding colony. Although we know little of the natural history of juvenile túngara frogs, they are sometimes found near breeding choruses thus learning in the wild would be possible.

In this study, we use rearing techniques and phonotaxis test to evaluate the role of early experience in the variation mate preferences, recognition, and discrimination of female *P. pustulosus.*

METHODS

Three hundred *Physalaemus pustulosus* tadpoles from 20 broods were equally divided into one of four acoustic rearing groups and maintained in these groups through metamorphosis to sexual maturity. The first rearing group was exposed to a natural chorus of male *Physalaemus pustulosus*. The second group was exposed to a natural chorus of a related species, *P. enesefae*. For both of these groups, the chorus was broadcast for the 12 hours corresponding to their night cycle. The third group heard no calls, only ambient sounds in the room. The fourth group heard broadband white noise for 24 hours each day. All frogs were kept in enclosures that were 32 cm wide, 60 cm long, and 18 cm tall, maintained at the same temperature and humidity with a 12-hour day/night light cycle. Frogs were fed *ad libitum*. All enclosures were sound attenuated by 35 dB from one enclosure to the next; sound levels between boxes were well below the threshold for this species at these frequencies (Wilczynski et al., 2001). Boxes were opened during the 12-hour day cycle to provide air circulation. There were four boxes (replicates) of each of the four rearing conditions, interspersed systematically in a four-by-four grid, for a total of 16 enclosures.

As each female reached sexual maturity (25 mm snout-vent length), she was tested in a series of phonotaxis tests to determine the degree to which she discriminated between a pair of calls or to which she recognized a single call that was paired with a white noise control. These tests were performed in a 2.7 x 1.8 meter acoustic chamber illuminated by ceiling mounted infrared lights and equipped with an infrared camera to allow undisturbed observations of the female behavior from a monitor outside the chamber. Each female was placed in the center of this arena and kept immobile under a funnel while two stimuli were played alternating through speakers at each side of the chamber at a sound level of 81 dB SPL (re: 20 μmPa). After three minutes, the female

was released and allowed to move freely. A female's behavior was defined as a choice if 1) she moved from the center within five minutes, 2) she continued to move at least every two minutes, or 3) she moved within 10 cm of a speaker within 15 minutes of being released. Females were injected with 250 IU of human chorionic gonadotropin 12-18 hours before being tested, to simulate the hormonal states of female *P. pustulosus* in the wild (Lynch et al., 2006). In this experiment, the dose was adjusted to account for the body size of each female. Observers for all female phonotaxis tests were unaware of the treatment each female received.

In contrast with the natural choruses used during rearing, calls used for phonotaxis tests were synthesized using the mean call characteristics of *P. pustulosus*, *P. enesefae,* or characteristics intermediate between the two. Calls of both species can be described by temporal and spectral characteristics (Figure 2). Temporal characteristics include the rise time, the time (in milliseconds) from the beginning of the call to the maximum amplitude of the call, and the fall time, from the maximum amplitude to the end of the call. In *P. pustulosus* calls, the rise time is typically very short relative to the fall time. Overall call duration is the sum of the rise and fall times. *P. enesefae* calls have a longer duration. Spectral features consist of the frequency at the initiation of the call and frequency at the end of the call. *P. pustulosus* and *P. enesefae* calls are similar in their spectral characteristics.

Females were tested for their preferences in up to nine stimuli pairs (Table 1). The general questions we addressed were: Do females show phonotaxis toward conspecific calls and do they prefer complex to simple complex calls? Do they falsely recognize non-conspecific calls as signaling an appropriate mate? At what signal to noise ratio is their release from masking? We then determine if treatment influenced the responses of females in each of these contexts.

All females were first tested for their preference for conspecific simple versus complex calls (consisting of a simple call followed by one chuck). Phonotactic response would show that females recognize the conspecific calls and the specific choice would show if they exhibited a preference for complex calls, as in the wild. This also screened for females who were receptive. Females who did not make a choice in this initial test were retested later that night; females who failed to make a choice that night were placed back into their rearing environment and retested three weeks later. Females were scored as "no choice" if they chose the noise or made no choice at all; females were scored as "choice" if they chose the simple call, the heterospecific call, or the intermediate call. The fifth test was always another test of the simple versus complex calls, and each female was required to make a choice in order for her choices in the prior tests to be included. Choices were scored as described above for the first test.

Females who responded when presented with the conspecific simple versus complex call were then tested for their recognition of various stimuli by pairing a call with a white noise control. We determined if females recognized the conspecific simple call (preference for conspecific complex calls in the previous experiment would not address this question), the heterospecific *P. enesefae* call, and a synthetic call intermediate between the *P. pustulosus* and *P. enesefae* call.

Females reared hearing the conspecific *P. pustulosus* chorus and the noise rearing groups were tested with additional stimulus pairs. Noise-masked simple calls were paired with the amplitude-modulated noise to test release from masking. The peak amplitude of the noise-masked calls were 6, 12 and 18 dB below the peak amplitude of the masking noise with the amplitude of the masked noise decreased so that each stimulus had an amplitude of 81 dB SPL. After these pairs were presented, in varying order, these females

received a final test of simple vs. complex calls. As before, only the results from females who successfully completed this final test were included.

Twenty-two females in each treatment group completed all of the appropriate phonotaxis tests. A logistic regression was used to test for the effect of early experience treatments and an interaction between treatments and replicate position for each phonotaxis test. An exact binomial test was used to test for choice preferences in each phonotaxis pair.

RESULTS

In all tests, there were no differences in the choices made based on the female's early experience and there was no interaction between early experience and replicate position (Table 3).

In the test of a simple conspecific whine paired with a complex conspecific whine-chuck, more females in chose the conspecific complex call (P=0.001; Figure 11). Of the 88 females tested, 84% chose the whine-chuck over the whine, a percentage that is statistically not difference from that seen in much larger datasets of female phonotaxis in this species (P=0.388) (Gridi-Papp et al., 2006). When presented with amplitude-modulated noise versus the simple conspecific call, females more often chose their own species call (P<0.001; Figure 12) and chose this simple call at a rate comparable to the preference shown in previous studies (P=0.281). When tested for their choice between noise and the intermediate call or *P. enesefae* calls, females significantly did not choose the intermediate call (P=0.002; Figure 13) or the *P. enesefae* (P<0.001; Figure 14).

Female *P. pustulosus* reared hearing the conspecific chorus and reared hearing noise were additionally presented with phonotaxis tests pairing amplitude-modulated noise with noise-masked conspecific simple calls. Females in both rearing groups were no more likely to choose the 6 dB masked call (P=0.542; Figure 15). This is significantly

different from the test pairing an unmasked whine with noise ($P<0.001$). Females in both groups did not choose calls masked with 12 dB of noise ($P<0.001$; Figure 16) or calls masked with 18 dB of noise ($P<0.001$; Figure 17).

DISCUSSION

Females reared in four acoustic groups made similar choices in all phonotaxis tests, indicating that when presented with specific early experiences, female choice was not altered. These results support the hypothesis that environment plays at minimum a limited role in the development of mate preferences in female *P. pustulosus*. Females recognized their own species advertisement call and preferred the conspecific complex call to the simple call, which was also found in previous studies of this species (Ryan, 1980; Ryan & Rand, 2003b; Gridi-Papp et al., 2006).

There were no effects of early experience on the ability of females to detect noise-masked calls. Exposure to constant noise did not improve females' ability to detect calls, even with 6 dB of noise masking. Females need to deal with noise from biological sources such as heterospecific anurans and abiotic sources such as waterfalls and wind. Future studies are needed to explore the threshold of masking noise with respect to actual conditions at mating sites.

Studies show that learning only rarely plays a significant role in the acquisition of female mate preferences. Even in songbirds, a model system for studies of acoustic communication in which male learning is well-studied, females of many species do not learn mate preferences although there are some conflicting results. Female cardinals do not show a preference for the songs of males to whom they were exposed during development (Yamaguchi, 1999). Female white-crowned sparrows respond almost exclusively to males singing their home dialect (Baker et al., 1981), although another study of the same species found no similar effect (Baptista & Morton, 1982). At present

there appears to be little consensus on the role of learning in female preferences in songbirds (Riebel et al., 2005). Even less data exists for learning in invertebrates, where one study showed that female wolf spiders alter their mate choices based on early experience (Hebets, 2003). The presence of preference learning in such diverse taxa as songbirds and female invertebrates offers the possibility that such learning may be widespread and suggests that more research is needed.

Learning may play an important role in speciation. Learned mate preferences could increase the rate at which premating isolation occurs (Irwin & Price, 1999). It might also contribute to receiver biases (Ten Cate & Rowe, 2007). In *Physalaemus pustulosus,* research has demonstrated the importance of receiver sensory biases in the coevolution of the mating system (Ryan et al., 1990). The data presented here that show female *P. pustulosus* do not learn their mate preferences provide additional support for the role of sensory biases as the main factor driving evolution of the male advertisement call in this species.

Chapter 4: Early experience influences evoked vocal responses in male *Physalaemus pustulosus*

SUMMARY

Signals used in mating behaviors may also function in male-male competition. The development of these signals in context of their use in intrasexual communication is not well studied. To study possible contribution of early experience, we reared Neotropical frogs, *Physalaemus pustulosus,* in four acoustic environments: 1) conspecific chorus, 2) acoustic isolation, 3) heterospecific chorus, and 4) noise. For the heterospecific chorus, we chose the calls of a closely related species, *P. enesefae*, with which *P. pustulosus* is sympatric in parts of its distribution. We measured the evoked vocal responses of these frogs in response to conspecific calls, heterospecific calls, and calls representing an intermediate between the two species. Males reared hearing the calls of the other species produced more calls overall and more complex calls in response to the call of that species. This shows that male evoked vocal responses are plastic and can be influenced by early experience. Previous studies have demonstrated that *P. pustulosus* in Panama do not exhibit this vocal response, suggesting that the effect was caused by the early experience.

INTRODUCTION

Signals used in mate recognition can also function in intrasexual competition (Andersson, 1994). The same signals used by females for mate choice and may be evaluated at the same time by males. For example, bird song contains syllables that both attract females and cause neighboring males to cede territory (Jarvi et al., 1980).

In male-male competition, males often simultaneously produce and respond to signals. Males may base their detection on proximity of neighbors or acoustic space.

Males are more likely to respond to nearby neighbors (Wagner, 1989b), in one case using call amplitude (Wilczynski & Brenowitz, 1988). Just as females use a complex mix of cues when they express mate preferences, males react to a complex mix of call characteristics when choosing which neighbors to which to respond (Greenfield & Rand, 2000). Males may be more selective in their response when congeners are present, to avoid responding inappropriately to heterospecifics (Gerhardt, 1994).

In response to conspecific signals, males often alter their own calls. Cricket frogs *(Acris crepitans)* increase their pulse rate when hearing nearby males (Wagner, 1989a). Túngara frogs *(Physalaemus pustulosus)* increase the complexity of their call by adding a suffix "chuck" (Ryan, 1985). In both of these cases, the change in call increases its attractiveness to females (Kime et al., 2004; Ryan, 1985). In cricket frogs a lower dominant frequency mating call is correlated with larger body size, so lowering the dominant frequency may be an attempt to increase apparent body size (Wagner, 1992).

In a well-studied Neotropical frog, *Physalaemus pustulosus,* males produce either a simple (whine) or complex (whine-chuck) advertisement call (Figure 1). The whine is an amplitude modulated, downward frequency-sweep. The escalation from simple to complex calls is facultative and females prefer the complex call (Rand & Ryan, 1981; Ryan, 1980). Frog-eating bats preferentially prey on males making complex calls (Tuttle et al., 1982; Ryan et al., 1982). Males typically choose to escalate to the complex call only when other nearby males are calling (Ryan, 1985).

The *Physalaemus* genus includes species distributed from Middle America through northern South America to the Pacific Coast west of the Andes. Most species are allopatric with *P. pustulosus* with the exception of *P. enesefae* which is sympatric with *P. pustulosus* in the llanos of Venezuela (La Marca, 1992). Male *P. enesefae* produce only simple calls, with a similar downward frequency sweep but a longer duration compared

to the call of *P. pustulosus* (Figure 3). To avoid the cost of breeding with heterospecifics, females in this area of sympatry discriminate against the calls of *P. enesefae* and they do so in a probabilistic fashion (Ryan et al., 2003). In addition, female *P. pustulosus* discrimonate against a number of synthetic calls that are intermediate between the conspecific call and the *P. enesefae* call. Their results show the point where female response reaches a specific level, so those synthetic calls can be used to test thresholds for female preferences.

The evoked vocal responses of male túngara frogs has been found to be similar to the responses of females (Ryan & Rand, 1998). Males increase overall calling when presented with conspecific simple calls and increase the production of complex calls when presented with complex calls. Males do not typically increase calling when presented with calls of *P. enesefae* but do increase calling in response to the calls of other, allopatric species (Bernal et al., 2007).

To date, few studies have examined the mechanisms that contribute to the development of male responses. Behaviors may be due to genetics, environment, or an interaction of the two. Early experience is a well described mechanism underlying variation in songbird calls (Thorpe, 1958) and female response to bird song (Miller, 1979). Male songbirds discriminately respond to local dialects but the function and development of this response is not known (Searcy et al., 1997).

In this study, we use evoked vocal response tests to evaluate the male-male interactions of male túngara frogs raised in four acoustic treatment groups to evaluate the relative contribution of early experience in the development of male response to conspecific signals.

METHODS

We reared three hundred *P. pustulosus* from 20 separate broods in sound-attenuated enclosures approximately 32 cm wide, 60 cm long, and 18 cm tall. Each brood was separated into one of four acoustic conditions: 1) *P. pustulosus* chorus, 2) isolated from all frog calls, 3) *P. enesefae* chorus, and 4) noise low-pass filtered at 5k Hz. Four enclosures received each acoustic condition (replicates), systematically interspersed in a 4x4 grid. All 16 enclosures were maintained in the same room. Speakers broadcast the playback for groups 1 and 3 at 80 dB SPL, the amplitude of a calling male measured at 1 meter, for 12 hours during each night photoperiod. The noise was also broadcast at 80 dB SPL but for 24 hours. All animals were maintained in these treatments from tadpole stage until sexual maturity (approximately 24 mm SVL, 8-10 months of age). They were fed ad libitum and received 12 hours of light each day. The enclosures did not have standing water present; this species will not call except from standing water, so the lack of such prevented males in the enclosures from calling.

At sexual maturity, we placed individual males in small sound-attenuated enclosures (22 cm wide, 24 cm long, 40 cm tall) and presented each male with a small pond of water from which to call. Males heard a chorus of *P. pustulosus* played from an Altec-Lansing VS2320 speaker driven by a Dell 2350 computer. Any vocalizations produced by the male were recorded on a Juster MP-018 microphone to a Dell 2320 computer. The sox sound utility was used to capture the sound files which were then evaluated using the CoolEdit (Syntrillium) software program. Males that called during an initial 30 minute period were then presented with the evoked vocal response experiments.

Each evoked vocal response experiment consisted of two stimuli. The first stimulus was played for one minute, followed by one minute of silence. The second stimulus was then played for one minute, followed by one minute of silence. Finally, the

first stimulus was played a second time for one minute. Each experiment lasted five minutes and was followed by a seven minute period consisting of one minute of silence, five minutes of a chorus of *P. pustulosus,* and one minute of silence. All experiments were performed in the small sound-attenuated enclosures and each playback was presented at 81 dB SPL. Male vocalizations during the evoked vocal response experiments were recorded on a Juster MP-018 microphone connected to a Dell 2320 computer and saved to digital sound files using the sox sound utility. The number of total calls produced during each stimulus section was counted manually using the CoolEdit software program. The number of chucks produced was also counted. Three independent and blind evaluators analyzed each sound file. Only males for whom all the evaluators' counts agreed were included in the study. Only experiments in which a male called at least once per minute were included.

The experiments are summarized in Table 4. Each male receiver four experiments, presented in systematically varying order. Each experiment is represented by the mean change in calls (the number of calls produced during the second stimulus minus the average number of calls produced during the first stimulus) and the mean change in chucks (the number of chucks produced during the second stimulus minus the average number of chucks produced during the first stimulus). All counts were corrected for the calling activity during silence.

The data were analyzed using a multivariate analysis of variance to test for effect of replicate position (SPSS 15.0). The data were then analyzed for main effects of treatment group using analysis of variance with a Tukey's post hoc test for pairwise comparisons.

RESULTS

Overall, there was a main effect of treatment on the change in calling rate and the change in the production of chucks in all tests (F=1.55, df=24, P=0.036; Table 5).

Males in all rearing groups increased their calling rate and the overall production of chucks when the *P. pustulosus* complex call was paired with the simple call (Figure 18). Males also increase the production of chucks when a *P. pustulosus* simple call was paired with amplitude-modulated noise (Figure 19).

The evoked vocal responses of the males differed between rearing groups in tests pairing noise with the intermediate call (Figure 20) and the call of *P. enesefae* (Figure 21). In the test that paired noise with the intermediate call, the number of calls produced by the males reared hearing the *P. enesefae* chorus was significantly different than the number of calls produced by the males reared hearing noise (P=0.025). The number of chucks produced by the males reared hearing the *P. enesefae* chorus was significantly different than the number of chucks produced by males reared hearing the conspecific chorus (P=0.006), the acoustically isolated males (P=0.023), and the males reared hearing noise (P=0.001).

In the test that paired noise with a call of *P. enesefae*, the *P. pustulosus* males reared hearing the chorus of *P. enesefae* produced more calls than did males reared hearing the conspecific chorus (P=0.010) and the males reared hearing noise (P=0.001). The difference in the number of calls produced between the males reared hearing *P. enesefae* chorus and the males reared in acoustic isolation was not significant (P=0.131). The number of chucks produced by males reared hearing the *P. enesefae* chorus when presented with a *P. enesefae* stimulus was significantly different than the number of chucks produced by the males reared hearing the conspecific chorus (P=0.017) or the calls reared by males hearing noise (P=0.014).

DISCUSSION

These results show that the response of male *P. pustulosus* to the calls of other males can be altered by early experience. In particular, male *P. pustulosus* reared hearing the calls of a related species, *P. enesefae,* were more likely to call, and more likely to produce complex calls, when they were presented with the *P. enesefae* call and a call intermediate between *P. pustulosus* and *P. enesefae*.

These results are in contrast to published data on the vocal responses of *P. pustulosus* studied in Panama in which males did not respond to the calls of *P. enesefae* (Bernal et al., 2007). However, that study and this one used males from Panama, a population that is not sympatric with congeners. In the llanos of Venezuela, *P. pustulosus* is sympatric with *P. enesefae*. Males in this area that exhibit this plasticity in their vocal response might be exposed to higher risk of predation. Future studies should look at possible population-level differences in the plasticity of vocal responses of male *P. pustulosus,* comparing individuals from areas of allopatry and sympatry for the influence of early experience on their vocal responses.

Male *P. pustulosus* do exhibit considerable plasticity in their vocal responses, such as facultatively escalating to complex calls in response to conspecific or even heterospecific calls. We show here that early experience may be a source for this variation.

Early experience appears to influence male behaviors but not female preferences in *P. pustulosus*. In a previous study, the preferences of female *P. pustulosus* were not influenced by early experience (Dawson & Ryan, 2007b). Male advertisement calls varied slightly with experience; males that were acoustically isolated produced shorter calls that were less preferred by females (Dawson & Ryan, 2007a). In this study, we show that experience influences how male receivers respond to signals. Female

preferences are not influenced by experience while male signals are and male responses appear to be the most affected. This may be due to stronger selection against female permissiveness and part of a larger trend of sex differences in receiver permissiveness (Bernal et al., 2007).

The tendency of experience to influence male vocal responses could also have implications for range expansion. New regions of sympatry with other species, combined with a heightened vocal response to those new species, could broaden the region where the energetic and predation costs are present.

In summary, male vocal responses change with early experience. Male experience with heterospecific calls increased the rate of calling response to those calls. Experience may influence acoustic communication in anurans and play a larger role in the phenotypic plasticity of these behaviors than previously understood.

Table 1: Call characteristics of male *Physalaemus pustulosus*

		P. pustulosus-reared males	Isolation-reared males	*P. enesefae*-reared males	Noise-reared males
Call duration (ms)	Mean	265.948	222.418	274.994	271.763
	SD	59.988	61.720	44.972	55.683
	SE	10.770	11.268	8.211	10.166
	Min	149.801	75.510	127.689	113.373
	Max	358.782	366.235	356.118	382.489
Rise time (ms)	Mean	35.655	26.795	36.485	30.908
	SD	33.987	28.061	37.19	36.459
	SE	6.205	5.123	6.790	6.657
	Min	7.797	6.649	4.762	6.733
	Max	121.907	124.600	114.075	145.786
Fall time (ms)	Mean	230.293	195.621	238.509	240.871
	SD	60.712	58.204	49.103	54.401
	SE	11.084	10.627	8.965	9.932
	Min	126.474	41.097	115.897	101.197
	Max	328.112	308.357	323.803	369.066
Initial frequency (Hz)	Mean	943.071	940.209	936.578	932.665
	SD	18.112	20.598	17.978	13.004
	SE	3.307	3.761	3.282	2.374
	Min	922.860	923.330	923.410	923.560
	Max	986.150	990.070	989.850	981.180

Final frequency (Hz)	Mean	483.268	497.93	498.594	498.594
	SD	37.335	43.085	31.089	31.089
	SE	6.816	7.866	5.676	5.676
	Min	401.430	419.580	442.570	442.570
	Max	561.760	568.910	552.560	552.560

Calls of male *Physalaemus pustulosus* reared in four treatment groups were analyzed for spectral and temporal characteristics. Descriptive statistics represent the mean, standard deviation (SD), standard error (SE), minimum value (Min), and maximum value (Max) of each characteristic for each group of males. Values of call characteristics for 30 males in each treatment group were included, and each characteristic for each male was represented by the average of 30 calls for that male.

Table 2: Statistical tests of calls of male *Physalaemus pustulosus*

Statistical test	Description	F	df	P
MANOVA	Main effect of rearing treatment group	1.814	15	0.031
MANOVA Between-groups	Call duration time (ms)	5.817	3	0.001 *
MANOVA Between-groups	Rise time (ms)	0.523	3	0.667
MANOVA Between-groups	Fall time (ms)	4.238	3	0.007 *
MANOVA Between-groups	Initial frequency (Hz)	1.961	3	0.124
MANOVA Between-groups	Final frequency (Hz)	1.356	3	0.260
Tukey post-hoc	Duration time			
	Isolation vs. *P. pustulosus* reared males			0.016 *
	Isolation vs *P. enesefae* reared males			0.002 *
	Isolation vs Noise reared males			0.005 *
Tukey post-hoc	Fall time			
	Isolation vs. *P. pustulosus* reared males			0.081
	Isolation vs *P. enesefae* reared males			0.018 *
	Isolation vs Noise reared males			0.011 *

Values describing the spectral and temporal characteristics of male calls were tested for a main effect of treatment group using a multivariate analysis of variance. For characteristics in which there was an effect of treatment, a Tukey post-hoc test was used to compare results between the four rearing groups. Values of call characteristics for 30 males in each treatment group were included, and each characteristic for each male was represented by the average of 30 calls for that male. Physalaemus pustulosus males were reared hearing a chorus of P. pustulosus, acoustic isolation, a chorus of P. enesefae, or broadband noise low pass filtered at 5 kHz.

Table 3: Female phonotaxis tests

Stimulus 1	Stimulus 2	Rearing group	Chose stimulus 2	Regression	Binomial (distribution)
P. pustulosus simple call	*P. pustulosus* complex call	*Pp*	18	X2 = 10.086	P<0.001
		Isol	19	df=12	(0.50)
		Pe	18	P=0.608	P=0.388
		Noise	19		(0.85)
Noise (amplitude-modulated)	*P. pustulosus* simple call	*Pp*	16	X2 = 18.112	P<0.001
		Isol	20	df=12	(0.50)
		Pe	19	P=0.112	P=0.281)
		Noise	18		(0.85)
Noise	Intermediate call	*Pp*	7	X2 = 11.503	P=0.002
		Isol	5	df=12	(0.50)
		Pe	7	P=0.486	
		Noise	10		
Noise	*P. enesefae* call	*Pp*	3	X2 = 7.984	P<0.001
		Isol	2	df=12	(0.50)
		Pe	2	P=0.786	
		Noise	3		
Noise	6 dB masked *P. pustulosus* simple call	*Pp*	11	X2 = 1.536	P=0.542
		Noise	8	df=4	(0.50)
			(n=44)	P=0.820	P<0.001)
					(0.85)
Noise	12 dB masked simple call	*Pp*	0	X2 = 6.257	P<0.001
		Noise	3	df=4	
			(n=44)	P=0.181	
Noise	18 dB masked simple call	*Pp*	1	X2 = 1.410	
		Noise	0	df=4	P<0.001
			(n=44)	P=0.843	
Average call	Duration 50 ms longer	Isol	10		P=0.023
		Pe	9		(0.50)
			(n=24)		
Average call	Duration 25 ms longer	Isol	7		P=0.999
		Pe	5		(0.50)
			(n=24)		
Average call	Duration 10 ms longer	Isol	7		P=0.839
		Pe	4		(0.50)

Female phonotaxis tests were analyzed for an effect of early experience rearing treatment and an interaction between treatment and replicate position using a logistic regression. All data were then analyzed for each test using a binomial exact test to test against a 0.50 distribution and/or a 0.85 distribution. The 0.50 distribution was used to determine if the female choices were statistically different from 50/50. The 0.85 distribution was used where appropriate to determine if the choices were statistically different from the choices of wild-caught female *Physalaemus pustulosus* who choose the conspecific complex call over the simple call 85.6% of the time in two-choice phonotaxis tests.

Table 4: Male evoked vocal response tests

Stimulus 1	Stimulus 2	Rearing Group	Mean Change in Calls	Mean Change in Chucks
P. pustulosus whine	*P. pustulosus* whine-chuck	*Pp*	1.18	2.40
		Isol	3.03	1.12
		Pe	3.05	4.00
		Noise	0.73	3.08
Noise	*P. pustulosus* whine	*Pp*	3.85	5.19
		Isol	-0.10	5.44
		Pe	2.23	5.83
		Noise	0.73	7.50
Noise	Intermediate call	*Pp*	1.53	6.40
		Isol	-0.98	7.83
		Pe	5.35	17.28
		Noise	-2.70	3.93
Noise	*P. enesefae* call	*Pp*	-8.25	-2.63
		Isol	-5.55	1.18
		Pe	0.38	5.65
		Noise	-10.25	0.36

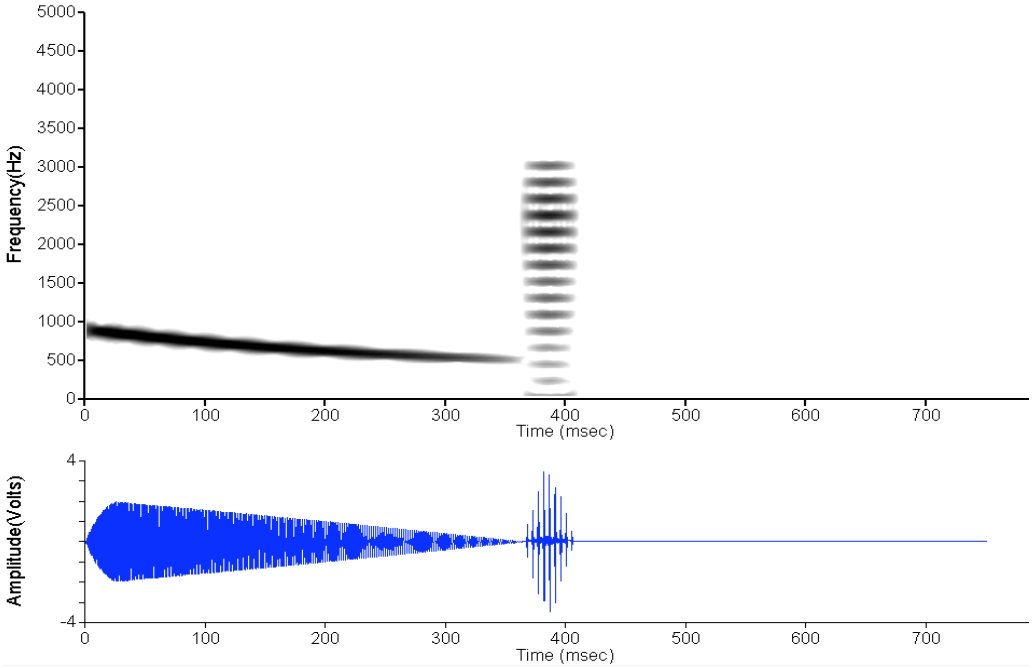
The number of calls and chucks produced during stimulus 2 were compared with those produced during stimulus 1 to produce a statistic of the mean change (increase or decrease) in calling and production of chucks.

Table 5: Statistical analysis of vocal response tests

Statistical test	Test Pair	Description	F	df	P
MANOVA	Overall	Effect of rearing group treatment	1.55	24	0.036
MANOVA Between-subjects effect	*P. pustulosus* complex vs simple call	Change in calling	0.851	3	0.470
		Change in chucks	0.804	3	0.496
MANOVA Between-subjects	Noise vs. *P. pustulosus* simple call	Change in calling	0.771	3	0.514
		Change in chucks	1.597	3	0.197
MANOVA Between-subjects	Noise vs. Intermediate call	Change in calling	3.161	3	0.029 *
		Change in chucks	6.570	3	0.001 *
MANOVA Between-subjects	Noise vs. *P. enesefae* call	Change in calling	5.915	3	0.001 *
		Change in chucks	4.268	3	0.008 *
Tukey post-hoc	Noise vs. Intermediate call	Change in calling			
		Pe-reared males			
		vs. *Pp*-reared males			0.518
		vs. isolation-reared males			0.113
		vs. noise-reared males			0.025 *
		Change in chucks			
		Pe-reared males			0.006 *
		vs. *Pp*-reared males			0.023 *
		vs. isolation-reared males			0.001 *
		vs. noise-reared males			
Tukey post-hoc	Noise vs. *P. enesefae* call	Change in calling			
		Pe-reared males			
		vs. *Pp*-reared males			0.010 *
		vs. isolation-reared males			0.131
		vs. noise-reared males			0.001 *
		Change in chucks			
		Pe-reared males			
		vs. *Pp*-reared males			0.017 *
		vs. isolation-reared males			0.360
		vs. noise-reared males			0.014 *

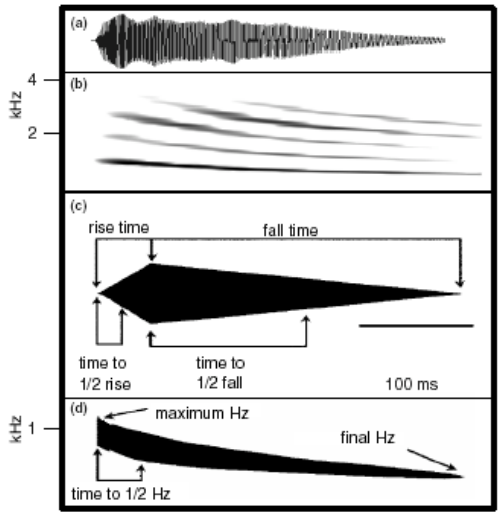
The number of calls and chucks produced during stimulus 2 were compared with those produced during stimulus 1 to produce a statistic of the mean change (increase or decrease) in calling and production of chucks. These were tested for an overall effect of rearing group treatment and between-subjects effects using a multivariate analysis of variance. For tests in which there was a between-subjects effect, a Tukey post-hoc test was used to further determine significant differences.

Figure 1: Advertisement call of male *P. pustulosus*



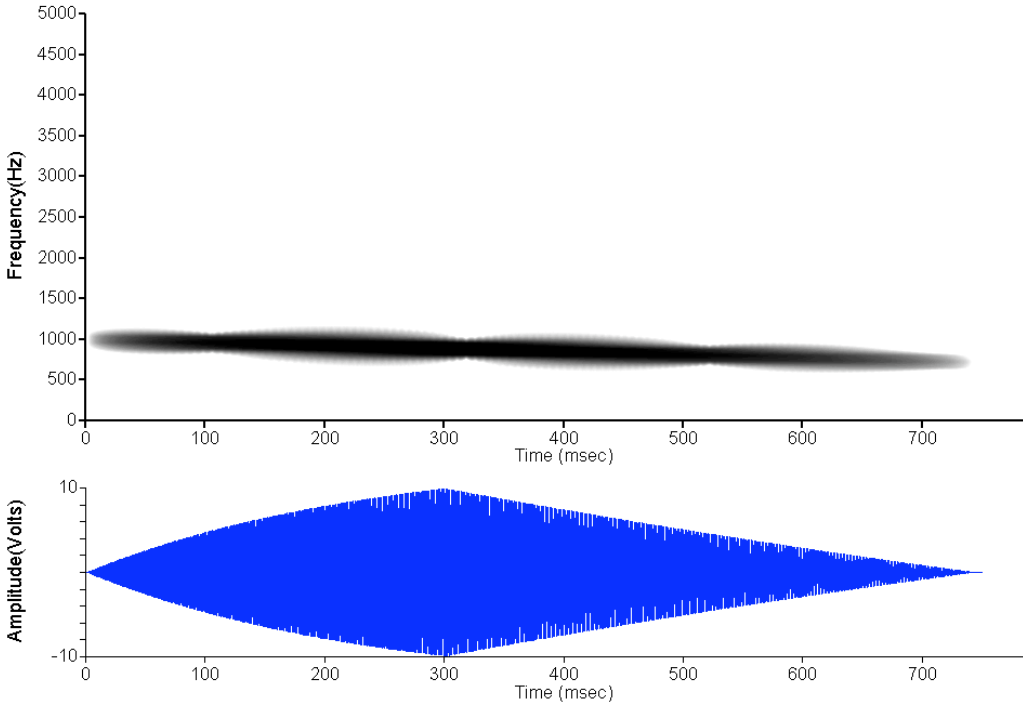
Male túngara frogs produce an amplitude modulated call that rises to maximum amplitude very quickly then decreases in amplitude to the end of the call. The frequency sweeps downward from an initial frequency near 1000 Hz and a final frequency near 400-500 Hz. When other males are calling, they often add a “chuck” at the end of the call, consisting of a short broadband sharply amplitude-modulated burst that increases the complexity of the call and its attractiveness to females.

Figure 2: Characteristics of the male advertisement call



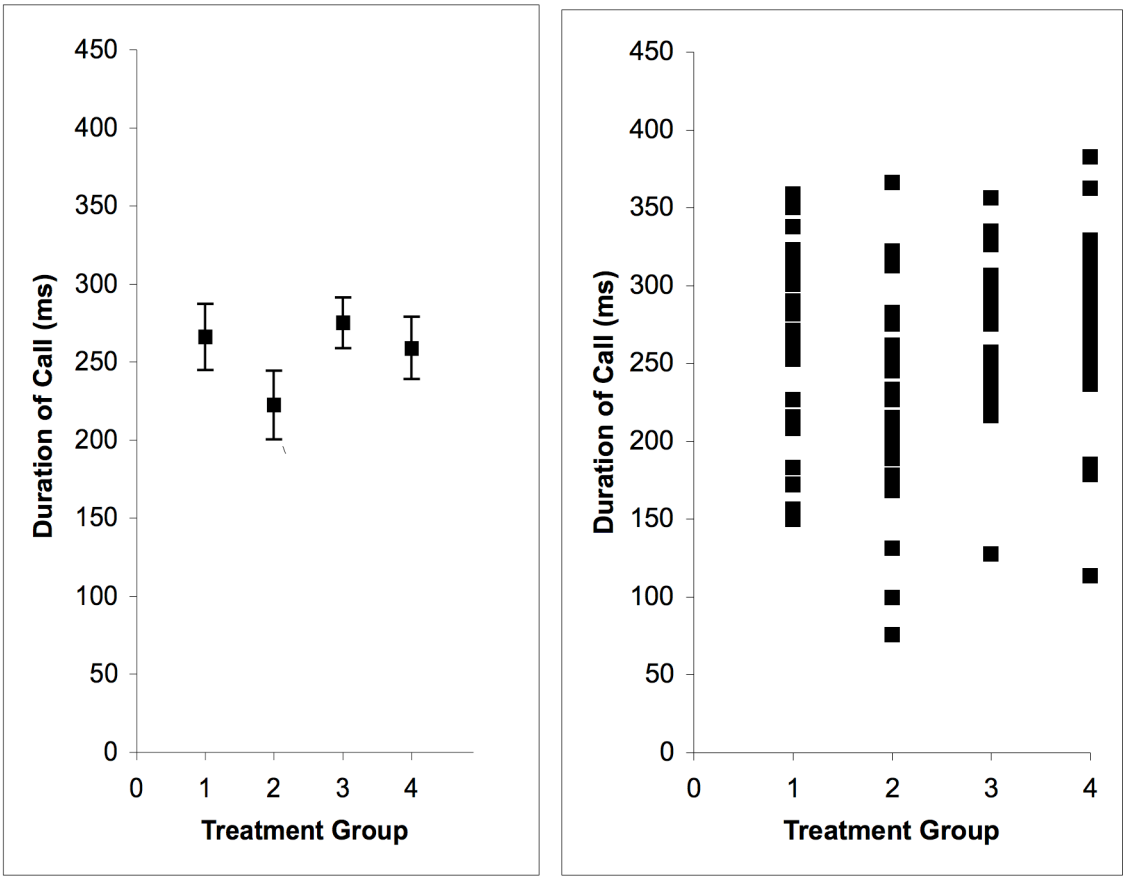
The advertisement call of male túngara frogs begins with a quick increase in amplitude (rise time) and a longer decrease in amplitude to the end of the call (fall time). Together, rise time and fall time comprise the overall call duration. The initial spectral frequency of the call is also typically the maximum frequency.

Figure 3: Advertisement call of male *P. enesefae*



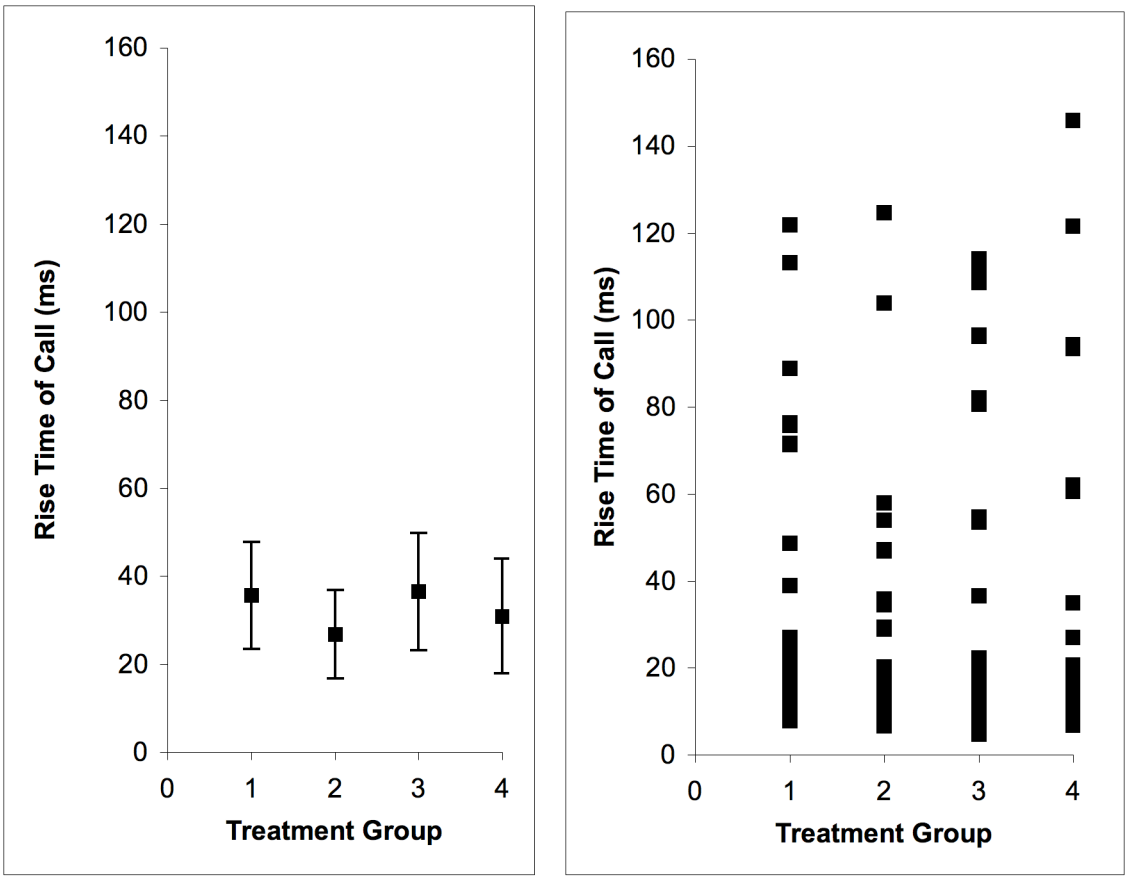
The advertisement call of male *Physalaemus enesefae* begins with a slow increase in amplitude to a maximum amplitude, followed by a gradual decrease in amplitude to the end of the call. In comparison to the call of the male *P. pustulosus,* this call has a longer time to maximum amplitude and a longer overall call duration.

Figure 4: The duration time of calls of male *Physalaemus pustulosus*



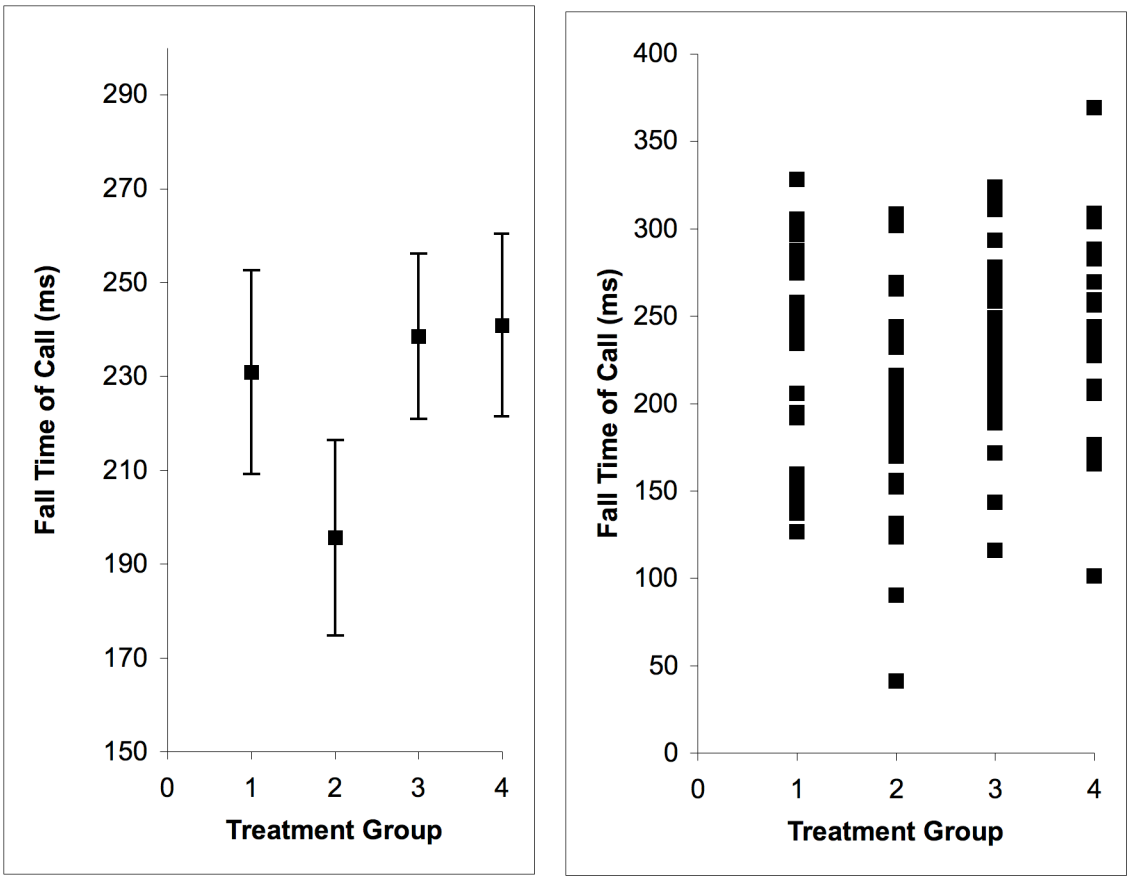
(Left) The mean duration of calls produced by male *Physalaemus pustulosus* reared in four early experience treatment groups (mean values with standard error bars). (Right) The range of duration times of calls for males in each group. The duration times of 30 calls for each male were averaged to produce a mean value for each male. Rearing groups consisted of: 1) a *P. pustulosus* chorus, 2) isolation, 3) a *P. enesefae* chorus, and 4) noise.

Figure 5: The rise time of calls of male *Physalaemus pustulosus*



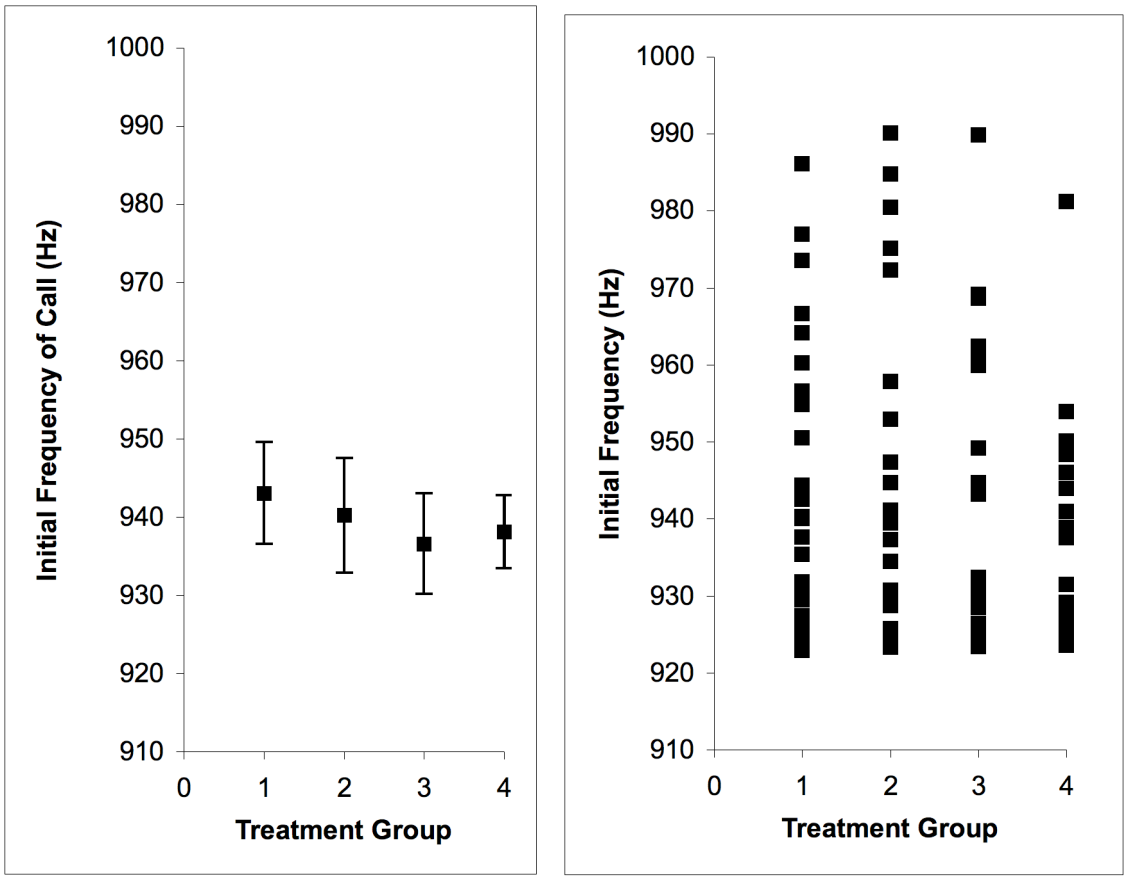
(Left) The mean rise time of calls produced by male *Physalaemus pustulosus* reared in four early experience treatment groups (mean values with standard error bars). (Right) The range of rise times of calls for males in each group. The rise time values of 30 calls for each male were averaged to produce a mean value for each male. Rearing groups consisted of: 1) a *P. pustulosus* chorus, 2) isolation, 3) a *P. enesefae* chorus, and 4) noise.

Figure 6: The fall time of calls of male *Physalaemus pustulosus*



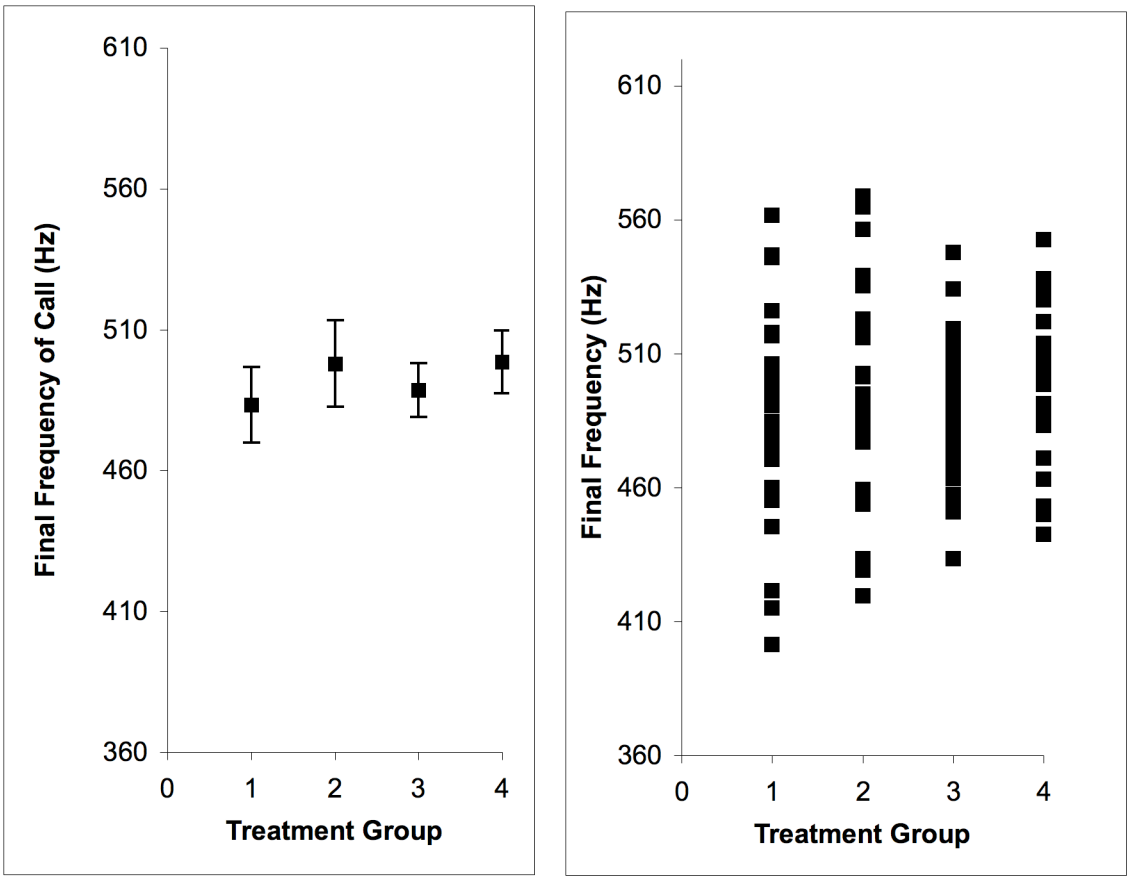
(Left) The mean fall time of calls produced by male *Physalaemus pustulosus* reared in four early experience treatment groups (mean values with standard error bars). (Right) The range of fall times of calls for males in each group. The fall time values of 30 calls for each male were averaged to produce a mean value for each male. Rearing groups consisted of: 1) a *P. pustulosus* chorus, 2) isolation, 3) a *P. enesefae* chorus, and 4) noise.

Figure 7: The initial frequency of calls of male *Physalaemus pustulosus*



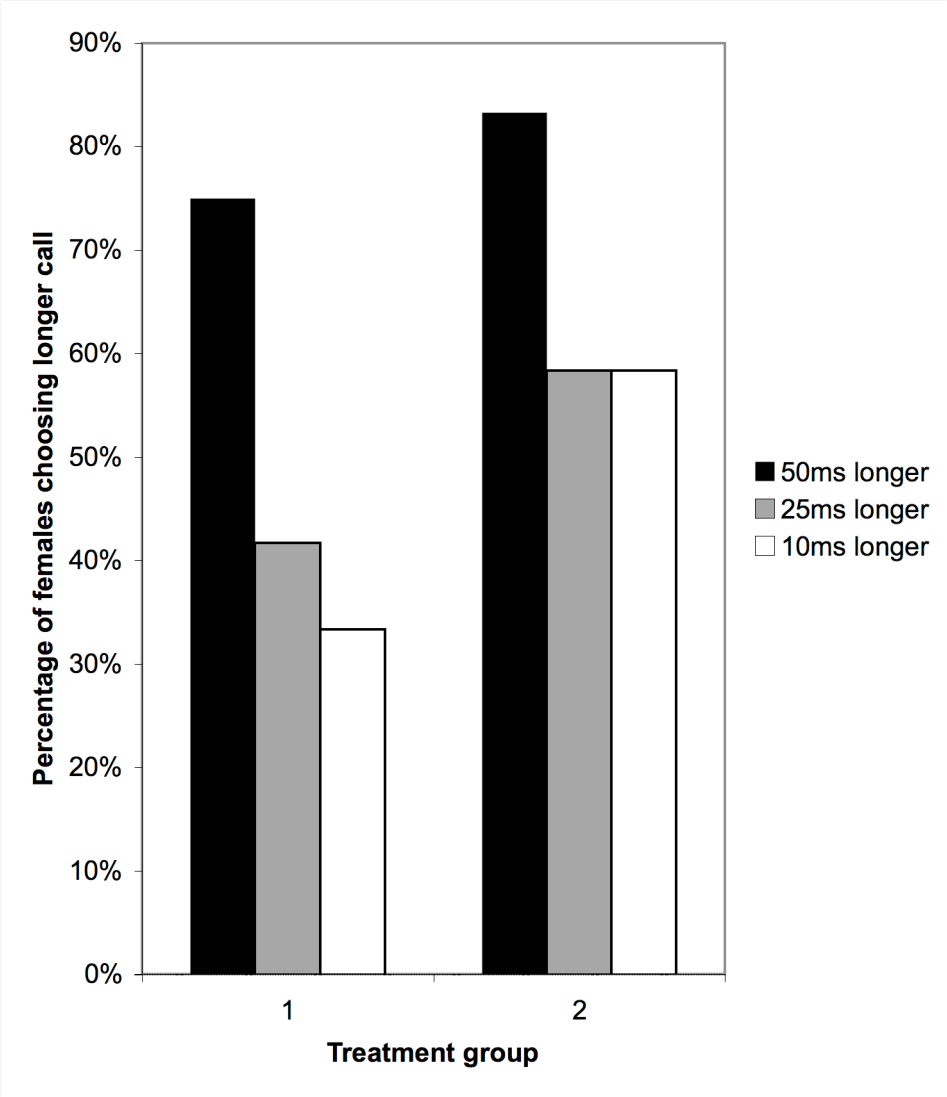
(Left) The mean initial spectral frequency of calls produced by male *Physalaemus pustulosus* reared in four early experience treatment groups (mean values with standard error bars). (Right) The range of initial frequencies of calls for males in each group. The values of 30 calls for each male were averaged to produce a mean value for each male. Rearing groups consisted of: 1) a *P. pustulosus* chorus, 2) isolation, 3) a *P. enesefae* chorus, and 4) noise.

Figure 8: The final frequency of calls of male *Physalaemus pustulosus*



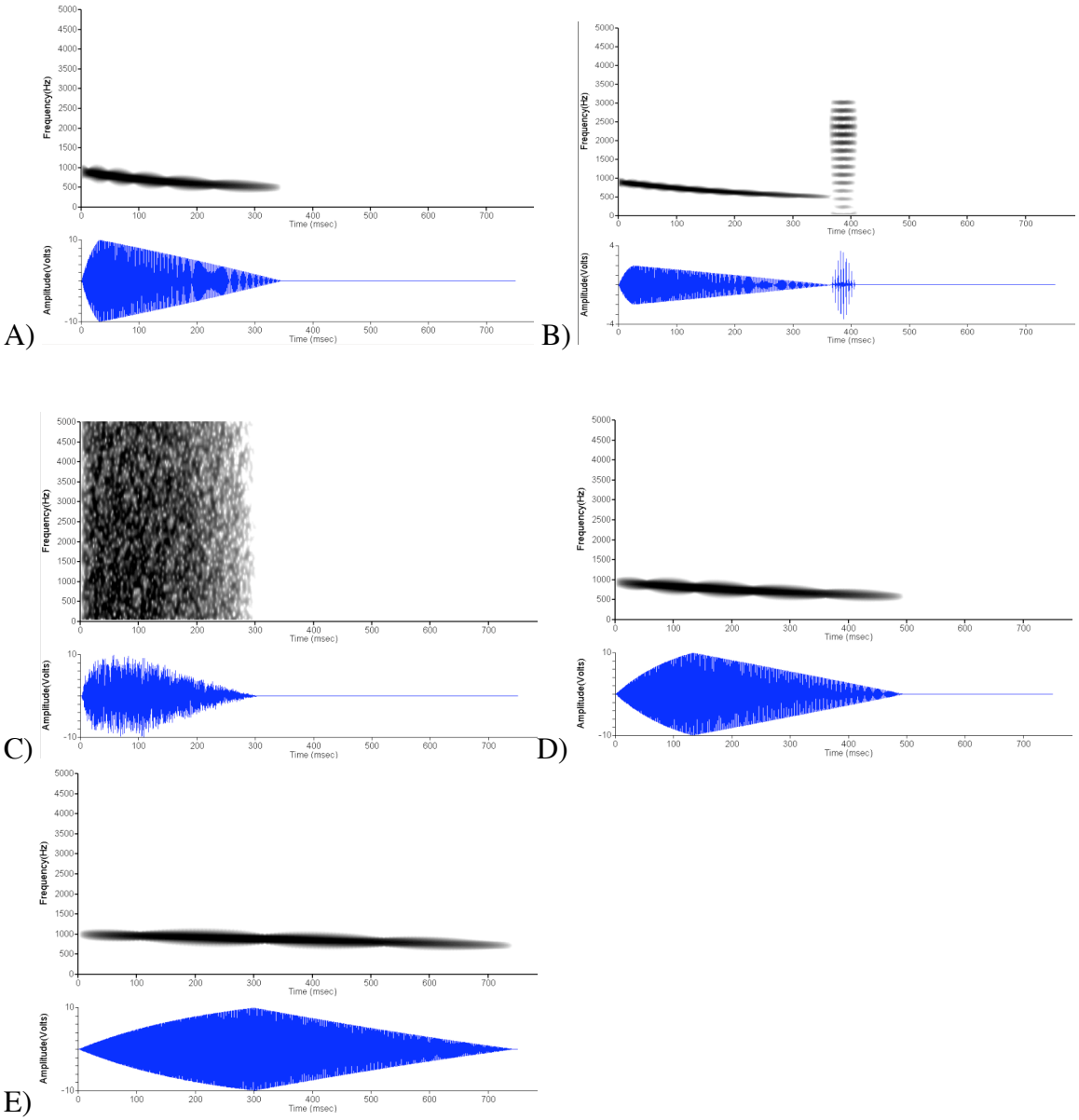
(Left) The mean final spectral frequency of calls produced by male *Physalaemus pustulosus* reared in four early experience treatment groups (mean values with standard error bars). (Right) The range of final frequencies of calls for males in each group. The values of 30 calls for each male were averaged to produce a mean value for each male. Rearing groups consisted of: 1) a *P. pustulosus* chorus, 2) isolation, 3) a *P. enesefae* chorus, and 4) noise.

Figure 9: Female responses to conspecific simple calls of varying duration



Females were more likely to choose a call that was 50 ms longer than a call of average duration (black) but that preference was not present in calls that were only 25 ms (grey) or 10 ms longer (white). Early experiment treatment groups were 1) *P. pustulosus,* and 2) acoustic isolation.

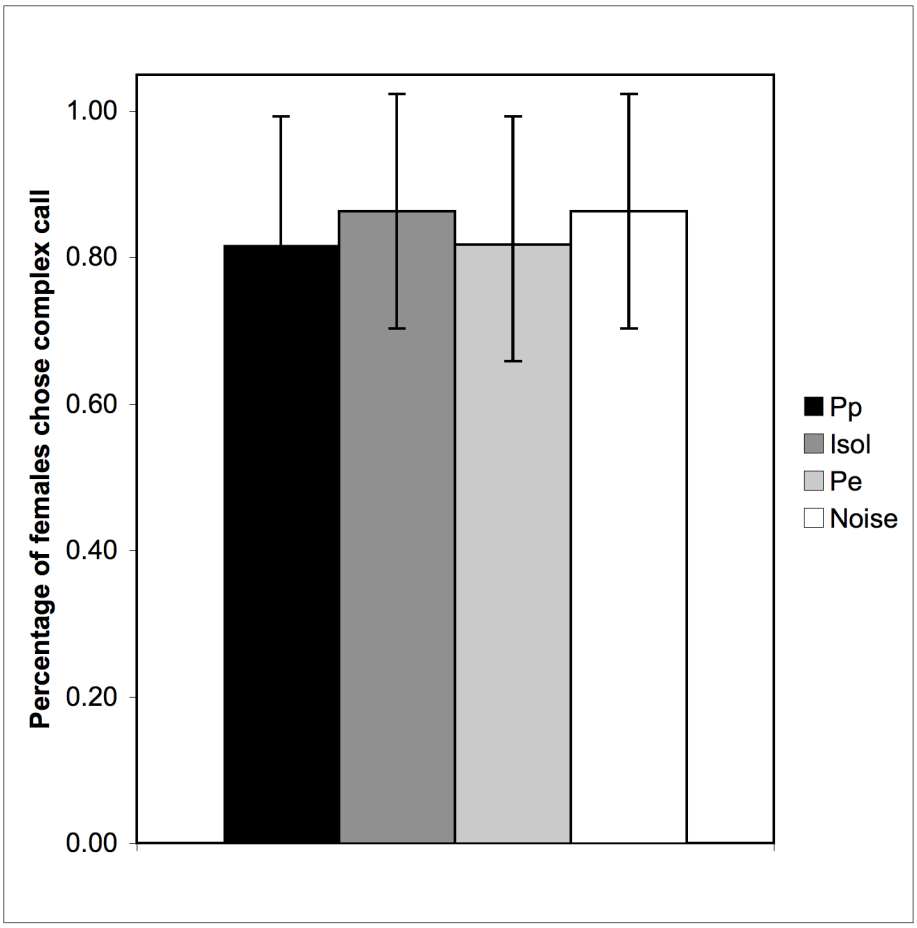
Figure 10: Stimulus files for female choice tests



Spectrograms (upper) and waveforms (lower) of five synthetic stimuli used to evaluate preferences of female *Physalaemus pustulosus* in two-choice phonotaxis tests: A) the simple call of conspecifics, *P. pustulosus,* B) the complex call of *P. pustulosus*, C) Noise

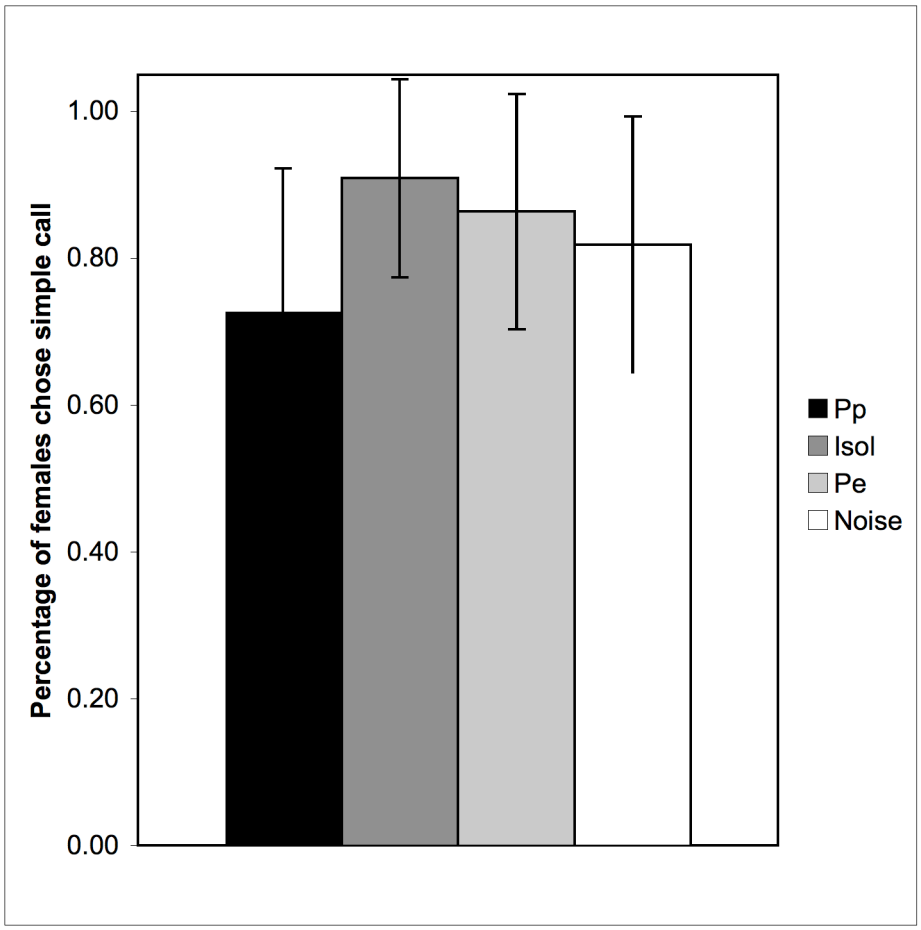
with the same amplitude envelope of a *P. pustulosus* simple call, but with no spectral structure, low-pass filtered at 5 kHz, D) a call intermediate to *P. pustulosus* and *P. enesefae,* and E) the call of the heterospecific *P. enesefae*.

Figure 11: Responses of female *P. pustulosus* to conspecific complex call



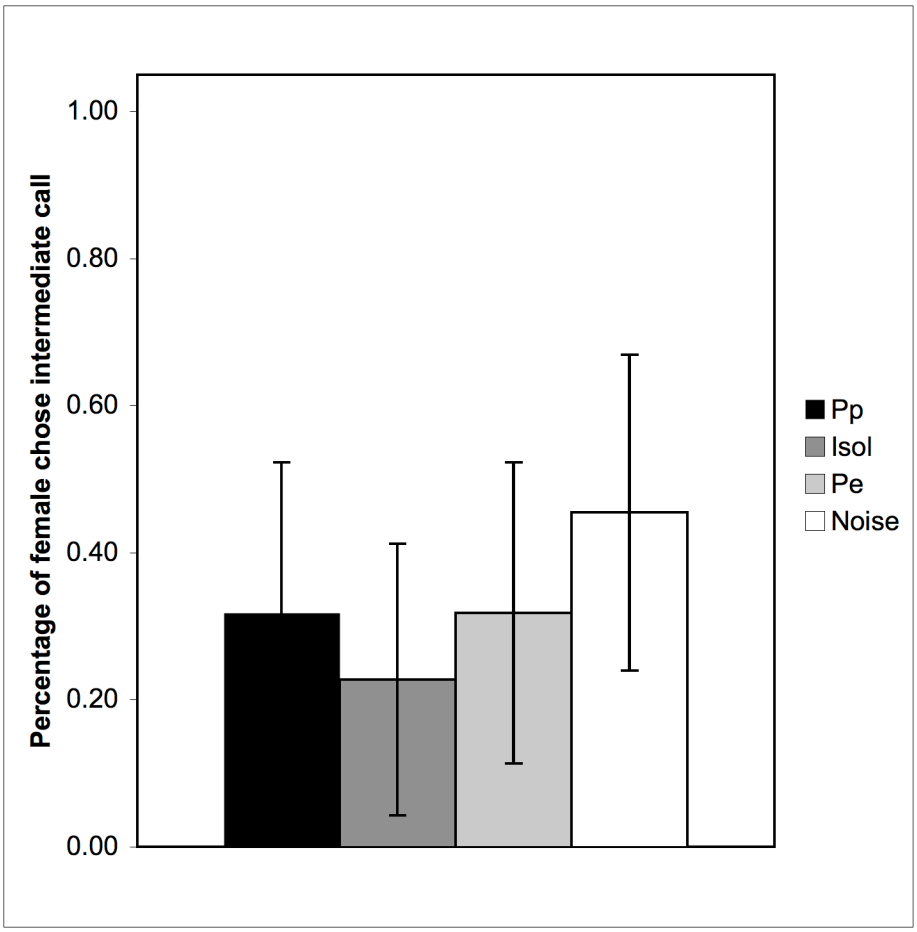
The percentage of female *P. pustulosus* who chose the conspecific complex call, paired with the conspecific simple call. Females were more likely to choose the complex call and chose that call at the same percentage as do wild-caught female *P. pustulosus*. There were no differences between the four early experience treatment groups (Pp = *P. pustulosus*, Isol = acoustic isolation, Pe = *P. enesefae,* and Noise = broadband noise low pass filtered at 5 kHz). Bars represent the 95% confidence intervals.

Figure 12: Responses of female *P. pustulosus* to conspecific simple call



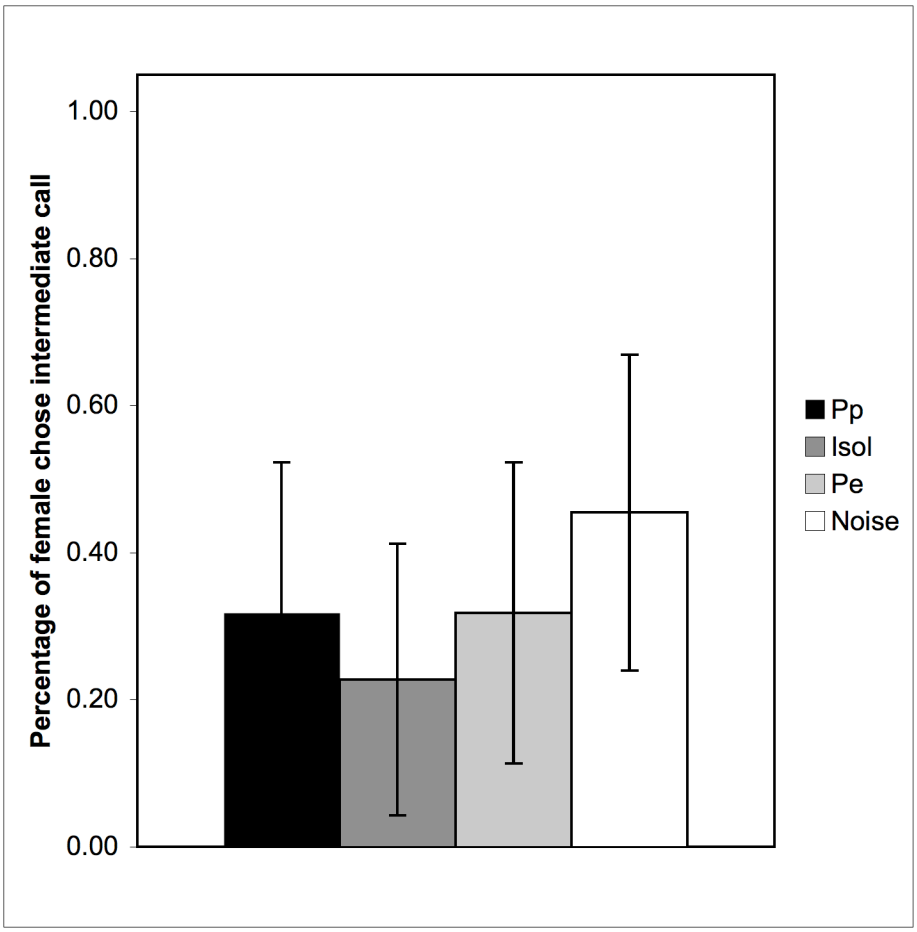
The percentage of female *P. pustulosus* who chose the conspecific simple call when paired with the amplitude-modulated noise. Females were more likely to choose the simple call. There were no differences between the four early experience treatment groups (Pp = *P. pustulosus*, Isol = acoustic isolation, Pe = *P. enesefae*, and Noise = broadband noise low pass filtered at 5 kHz). Bars represent the 95% confidence intervals.

Figure 13: Responses of female *P. pustulosus* to an intermediate call



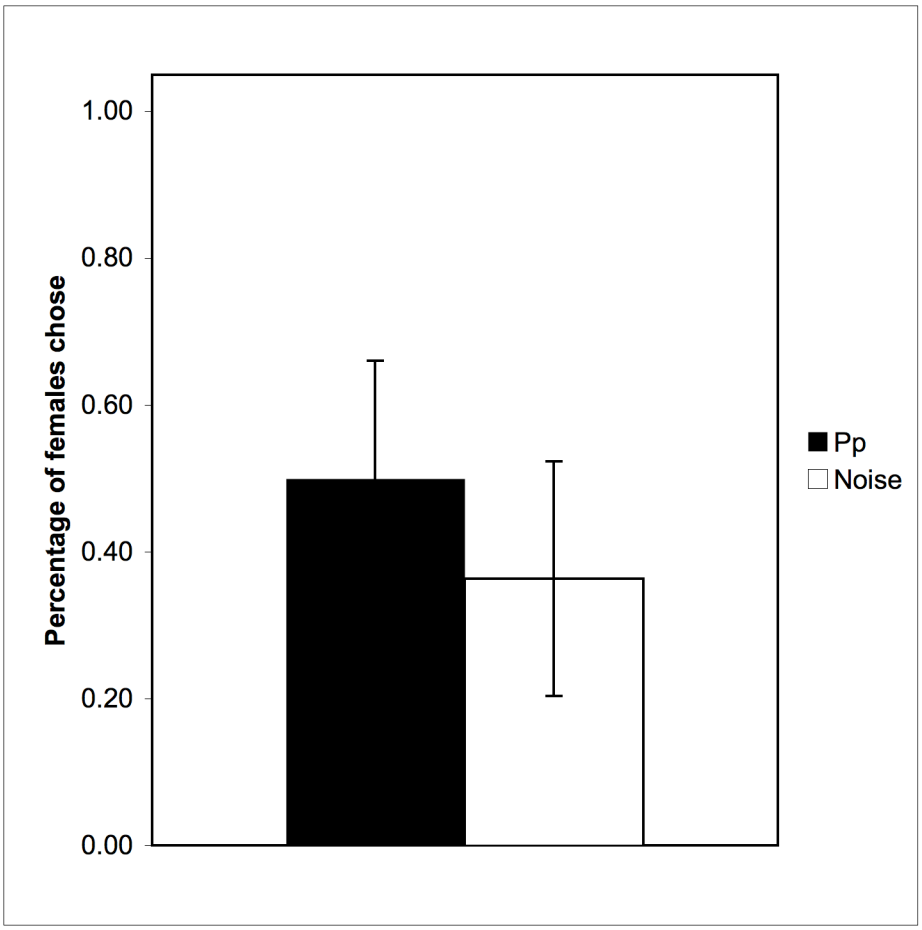
The percentage of females who chose the call intermediate between *P. pustulosus* and *P. enesefae* when paired with the amplitude-modulated noise. Females were not likely to choose the intermediate call. There were no differences between the four early experience treatment groups (Pp = *P. pustulosus*, Isol = acoustic isolation, Pe = *P. enesefae*, and Noise = broadband noise low pass filtered at 5 kHz). Bars represent the 95% confidence intervals.

Figure 14: Responses of female *P. pustulosus* to the *P. enesefae* call



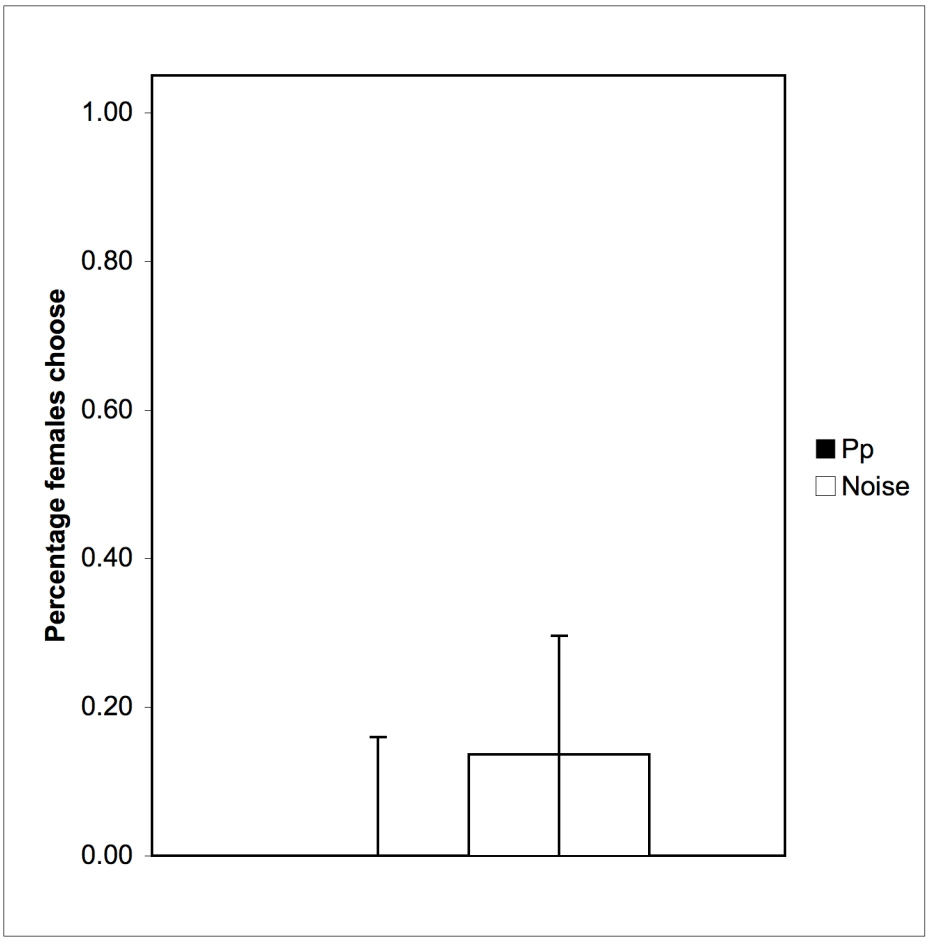
The percentage of *P. pustulosus* females who chose the *P. enesefae* call when paired with the amplitude-modulated noise. Females were not likely to choose the heterospecific call. There were no differences between the four early experience treatment groups (Pp = *P. pustulosus*, Isol = acoustic isolation, Pe = *P. enesefae*, and Noise = broadband noise low pass filtered at 5 kHz). Bars represent the 95% confidence intervals.

Figure 15: Responses of female *P. pustulosus* to the 6 dB masked call



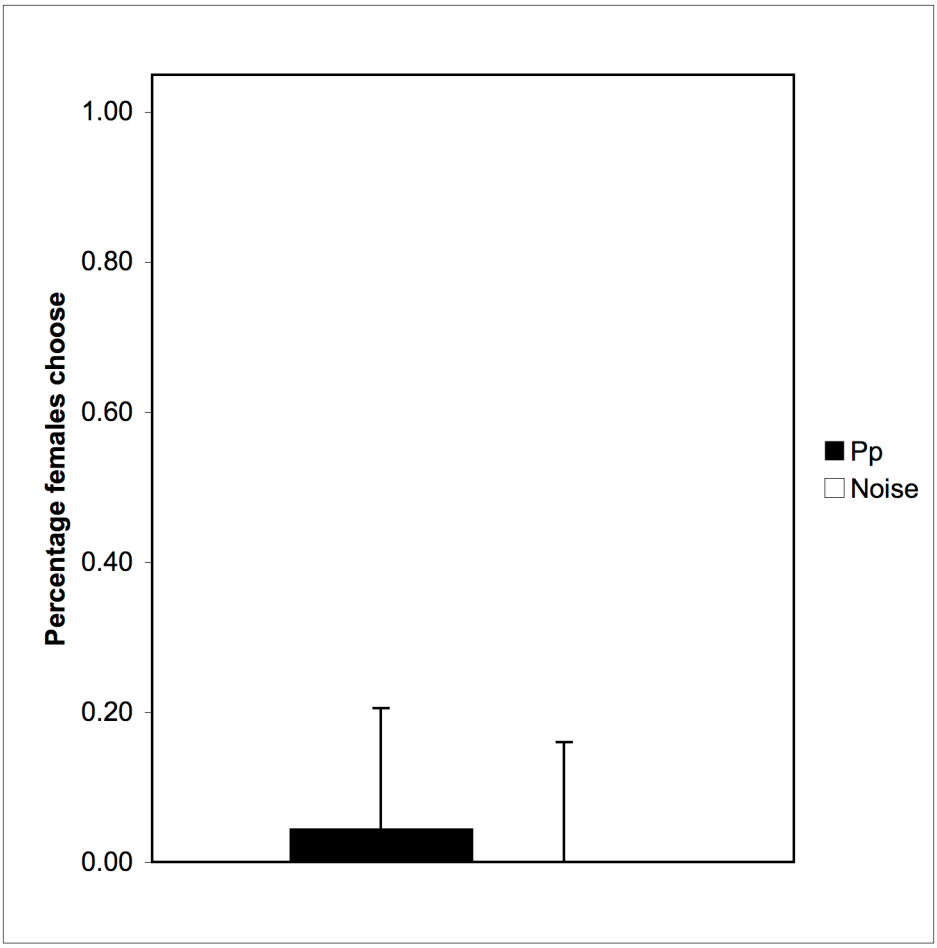
The percentage of *P. pustulosus* females who chose the conspecific simple call presented 6 dB below masking noise. There were no differences between the two early experience treatment groups (Pp = *P. pustulosus,* and Noise = broadband noise low pass filtered at 5 kHz). Bars represent the 95% confidence intervals.

Figure 16: Responses of female *P. pustulosus* to the 12 dB masked call



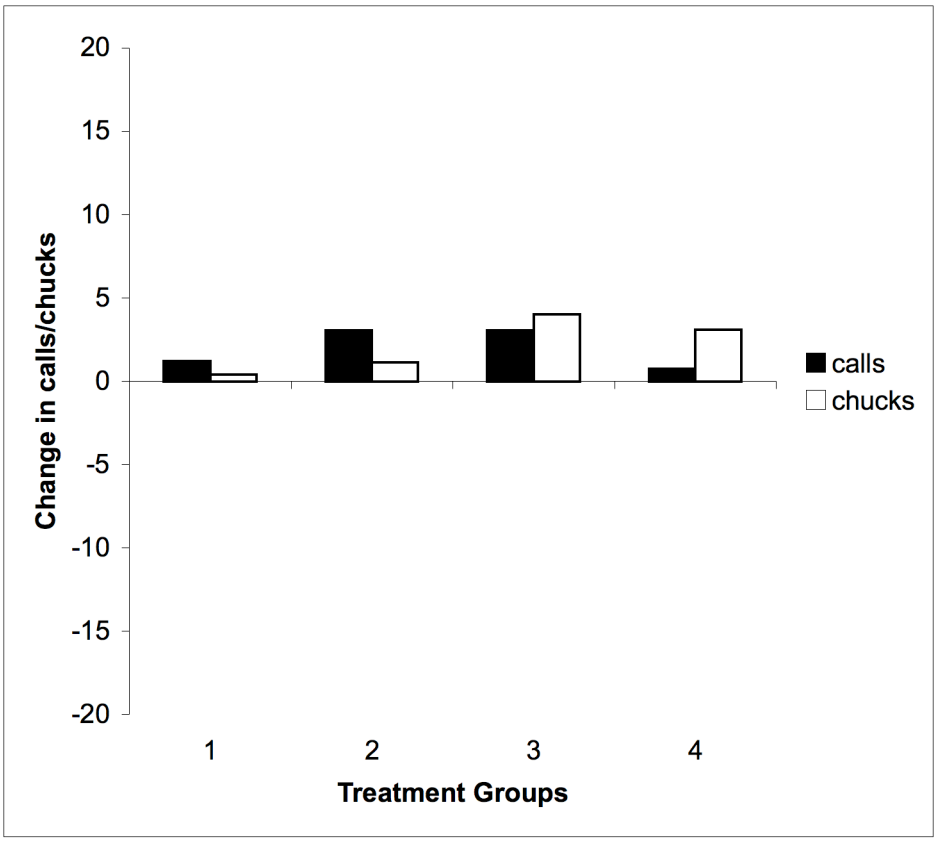
The percentage of *P. pustulosus* females who chose the conspecific simple call presented 12 dB below masking noise. No females in the *P. pustulosus*-reared group chose the masked call. There were no differences between the two early experience treatment groups (Pp = *P. pustulosus,* and Noise = broadband noise low pass filtered at 5 kHz). Bars represent the 95% confidence intervals.

Figure 17: Responses of female *P. pustulosus* to the 18 dB masked call



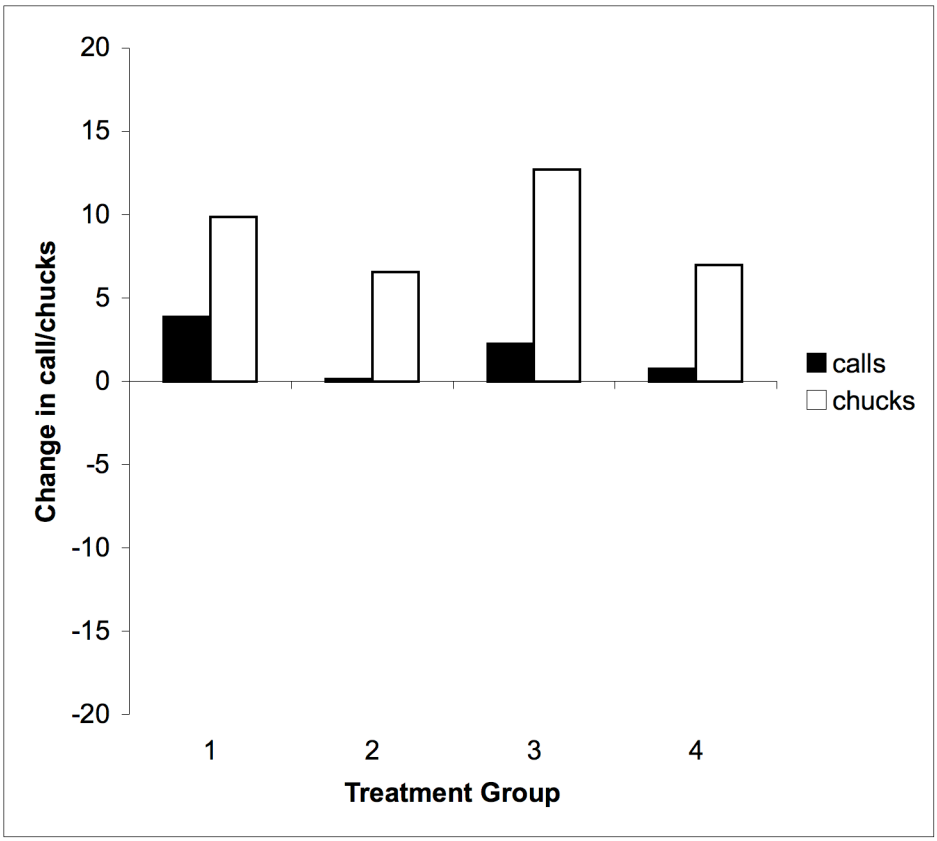
The percentage of *P. pustulosus* females who chose the conspecific simple call presented 18 dB below masking noise. No females in the noise-reared group chose the masked call. There were no differences between the two early experience treatment groups (Pp = *P. pustulosus,* and Noise = broadband noise low pass filtered at 5 kHz). Bars represent the 95% confidence intervals.

Figure 18: Vocal responses of male *P. pustulosus* to conspecific complex calls



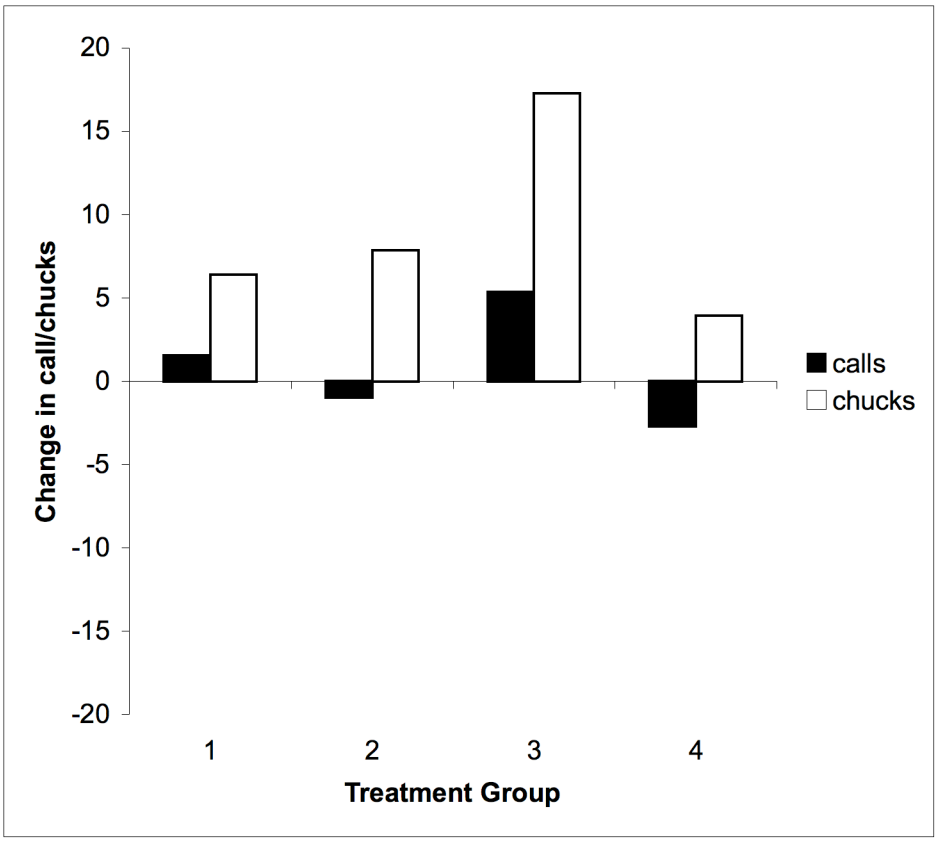
Males in all rearing groups produced more calls (black) and more chucks (white) when they were presented with the *P. pustulosus* whine-chuck complex call, relative to their responses to the *P. pustulosus* whine-only simple call. Rearing groups consisted of: 1) a *P. pustulosus* chorus, 2) isolation, 3) a *P. enesefae* chorus, and 4) noise.

Figure 19: Vocal responses of male *P. pustulosus* to conspecific simple calls



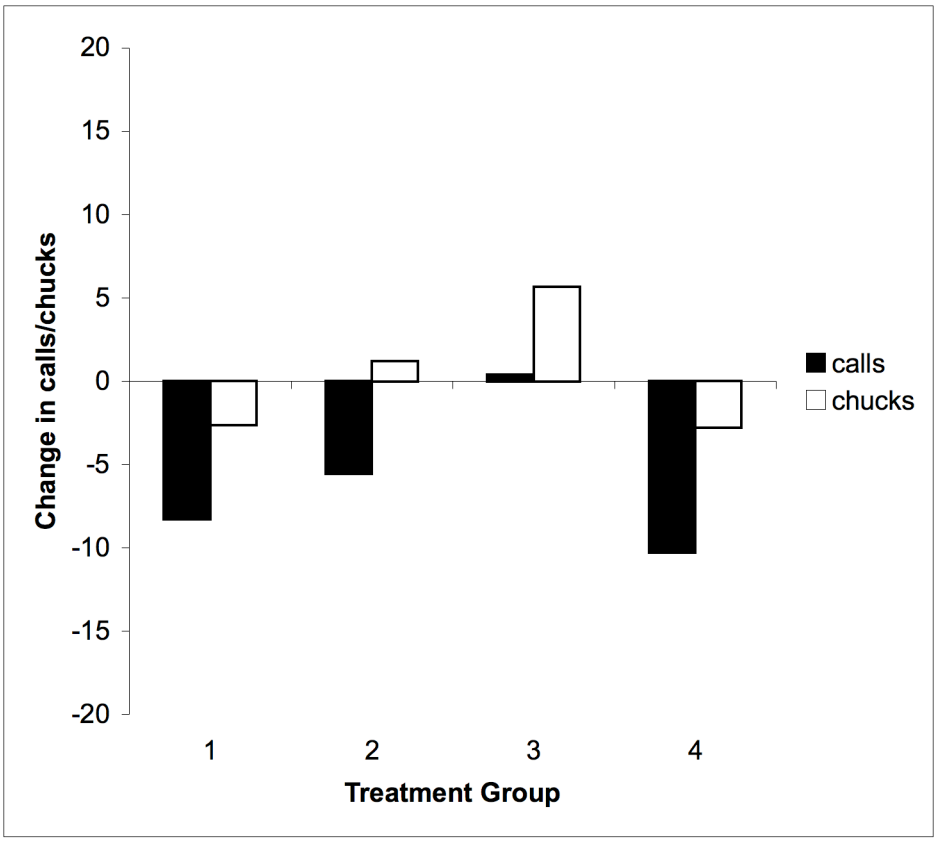
Males in all rearing groups produced more calls (black) and more chucks (white) when they were presented with the *P. pustulosus* simple call, relative to their responses to amplitude-modulated noise. Rearing groups consisted of: 1) a *P. pustulosus* chorus, 2) isolation, 3) a *P. enesefae* chorus, and 4) noise.

Figure 20: Vocal responses of male *P. pustulosus* to an intermediate call



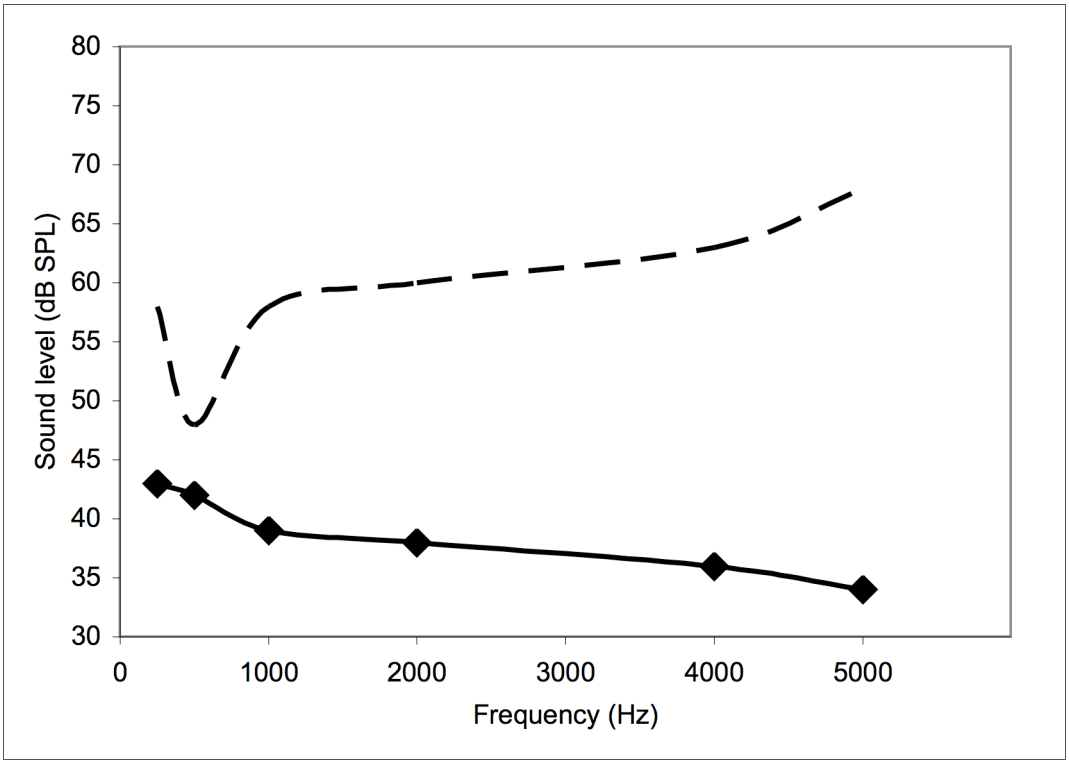
Male *P. pustulosus* in all rearing groups differed in their change in calls (black) and produced more chucks (white) when they were presented with intermediate call, relative to their responses to amplitude-modulated noise. Rearing groups consisted of: 1) a *P. pustulosus* chorus, 2) isolation, 3) a *P. enesefae* chorus, and 4) noise.

Figure 21: Vocal responses of male *P. pustulosus* to the *P. enesefae* call



Male *P. pustulosus* in three rearing groups reduced the production of calls (black) when presented with calls of *P. enesefae*, relative to their calling activity to amplitude-modulated noise. Two of the four groups produced more chucks (white) when they were presented with congener call, relative to their responses to amplitude-modulated noise. Rearing groups consisted of: 1) a *P. pustulosus* chorus, 2) isolation, 3) a *P. enesefae* chorus, and 4) noise.

Figure 22: *P. pustulosus* hearing sensitivity and sound levels in enclosures



The auditory system of *P. pustulosus* is more sensitive to sounds at frequencies matching those of the male advertisement call (500-1000 Hz) than it is to sounds at higher frequencies (dotted line). The sound levels inside a rearing enclosure (with the adjacent box playing tones at 81 dB SPL) are lower than the threshold of hearing of *P. pustulosus* at all frequencies (solid line).

Appendix A: Sound Attenuating Enclosures

Attenuation is the decrease in amplitude of a signal that typically occurs with distance and environmental impedance. For example, a sound wave attenuates as it propagates away from the source through the transmission medium.

In the research presented here, sound waves were deliberately attenuated to create acoustically isolated enclosures where frogs were reared. The enclosures were also designed to meet the requirements of animal husbandry, to be affordable, and to fit in the laboratory space available.

Dense materials provide the simplest way to attenuate sound waves. Sound waves of different frequencies will attenuate differently according to the density and width of the materials used. Materials such as a medium-density fiberboard provide substantial density and weight, but there are other considerations when choosing building supplies. Most materials resonate at a given frequency. For example, brass is dense, but its resonance properties make it an ideal choice for the construction of loud bells. As in most metals, the molecules in brass are connected in a regular lattice pattern that produces this resonance property. Materials with disassociated particles are a better choice for sound attenuation. Sand and particleboard are excellent attenuators because they are dense and the particles are relatively independent; sound waves do not propagate well through them.

For these experiments, I used two types of materials to maximize the efficiency of the attenuation from one enclosure to the adjoining enclosures. The walls in each enclosure were built with 0.5 inch sheetrock on the inside and 0.75 inch asphalt sheathing on the outside. The inner and outer walls were separated by a wooden frame made of 2 x 4-pine lumber. By using two materials of different widths and densities, I maximized the attenuation of sound waves in the frequencies of interest (500-1500 Hertz). The

enclosures were assembled using construction glue and drywall screws. Front-opening panels were custom cut for each enclosure to create a snug-fitting door. To test the attenuation, I played pure-frequency tones at 81 dB SPL in one box and recorded the sound levels in the adjacent box (Figure 22). At all frequency (from 250 to 5000 Hz), the sound levels inside the adjacent box were lower than the threshold of hearing for that frequency of *P. pustulosus*.

Each enclosure had an independent sound and light source, with the necessary cables routed through the lumber joints. The power and sound cables were threaded through the rear wall joints before the panels were put together. Each enclosure contained both LED and incandescent lights plugged into a power strip; the power strips for all the enclosures were controlled by a single timer. The sounds for each enclosure were played on Altec-Lansing speakers connected to a Dell computer running the Linux operating system. The technical specifications for the computer playback system are discussed in Appendix B. No behavioral data was obtained while the frogs were in these large enclosures.

Frogs were kept in separate plastic tubs within each sound-attenuating enclosure. Each tub was approximately 32 cm wide, 60 cm long, and 18 cm tall. The plastic tubs were sealed across the top with screen mesh attached to the tub with Velcro. The tubs created a secondary enclosure for the frogs that could be removed and cleaned in accordance with IACUC requirements for animal husbandry. The screen mesh tops allowed the sounds broadcast in each enclosure to reach the entire enclosure, and at the same time, keep the frogs (and fruit flies) secure. Each plastic tub housed approximately 30 adult frogs, the same density used to house frogs in the túngara frog colony at The University of Texas at Austin.

In the sound recording enclosures where male calls and vocal responses were recorded, space was less of an issue because only one animal was housed at a time and only for a brief period each night. Medium density fiberboard was used to create boxes large enough for one small plastic cage, with a screen "shelf" upon which to rest the speaker. The recording and playback systems used in these boxes are discussed in Appendix B. These boxes were constructed by the metal and woodworking shop at The University of Texas at Austin. The joints were glued together and the door was hung with a piano hinge. Dense foam and a fold-over latch secured the door. The test enclosures were much heavier and more expensive than the large enclosures that housed frogs.

Although the enclosures were sufficient for this experiment, a number of improvements could be made if similar ones are required for other projects. The outer wall of the large enclosures should be made of high-density fiberboard, a more expensive option than the asphalt sheathing, but one that would have more completely attenuated low frequencies. The double-walled construction used for the enclosures should be supplemented with sand so that the spaces between the sheetrock and the sheathing were filled. However, each option would substantially increase the weight of an enclosure. In addition, because sound waves travel through any crack or weak point in the construction, the attenuation of the enclosure is only as good as the attenuation of its weakest point. An enclosure that has sand-filled walls on five sides, but only has a thin door on the sixth side, will have little attenuation because the sound waves will propagate through the door. Constructing a double-walled door filled with sand is possible, but difficult.

Another solution is to house each enclosure in a separate room, but this tactic raises questions of pseudoreplication. If one room has environmental properties that are

not replicated in the other rooms, behavior could be affected in ways that confound the experimental results.

Barium-loaded vinyl is another construction material that should be considered. This material is very dense, relatively affordable, and easy to cut, glue, and nail. In this project, it could have been used between the sheetrock and asphalt sheathing to provide a much stronger attenuation. However, the vinyl has a strong odor and continues to outgas for years. A piece stored in an office drawer continued to have a strong and noxious odor two years after it had been placed there. Although this material might be acceptable for frogs, testing it would be required to ensure that the odor is safe and will not affect their behavior. Any enclosure must consider the requirements of the Animal Resources Center and the IACUC protocol for safe and effective animal housing.

Sound-attenuating enclosures can be constructed on a reasonable budget and with easily available construction materials. The process of constructing enclosures should begin with a thorough understanding of the frequencies that must be attenuated and the husbandry requirements of the research project.

Appendix B: The Use of Computers for Automating Experimental Procedures

Computers were used in these experiments in a variety of ways. Traditional uses of computers that were used included the analysis of digital sound files using software such as CoolEdit and Signal. More innovative uses of computers included using Linux command files to coordinate playbacks and recordings.

CoolEdit, unsupported since its sale from Syntrillium to Adobe (who has repurposed it for other uses), is a valuable software tool for visualizing files, generating simple playback files, and doing basic sound editing and analysis. It is an easy to use software program for Windows operating systems and can be learned by novices. In this research, we used CoolEdit manually to identify male calls (see Chapter 2) and to count responses in vocal response trials (Chapter 4).

Signal is a more expensive software problem that is more difficult to use, but powerful. The ability to write programs that manipulate and analyze files in batch mode is especially useful. For example, Signal can quantify the temporal and spectral features of tens or hundreds of prepared recordings. The Ryan Lab has scripts that analyze the temporal and spectral features of *Physalaemus* calls; these data are published in numerous papers on male call characteristics (Ryan & Rand, 2003a; Ryan et al., 2003). Automating this process not only saves time but also ensures that results are consistent. Signal's scripting language, an unfortunate blend of Basic and FORTRAN, is not accessible to novice users. A better choice for someone starting out in sound analysis is the Matlab environment, which is available as an open source alternative called Octave. If programmability and batch mode operation are not required, the Raven program from

Cornell's Lab of Ornithology is a happy combination of CoolEdit's ease of use and Signal's rigorous attention to detail.

Computers were also used to play sound files for the acoustic treatments in the rearing enclosures and in the testing enclosures for male call and male evoked vocal response tests. Computers were also used to record sound files during the male experiments. Dell computers ran the Linux operating system (RedHat Linux 9.0). There are a number of advantages to using Linux, including better security, availability of sound tools, and robust uptime. The computers were in use 24 hours a day, for more than three years without a single failure or unplanned reboot. The Linux operating system is scriptable; programs can be written to automate almost every process from synchronizing date and time across a network, to recording sound files, to saving input from sound cards. One disadvantage to Linux is the learning curve. Users familiar with the graphical user interfaces provided by Macintosh OS and Windows will need to invest some time to learn it; unfortunately, there are few end-user resources for technical support.

The scripts used in this experiment relied mostly on "crontab," the chronological scheduling function provided with Linux, and "sox," an open source sound processing program. Crontab was used to schedule sound playback in the enclosures and in the sound recording boxes. Crontab can be configured to start and stop playback on a 24-hour schedule. Sox was used to record the sound files. Invoked from command-line scripts, sox took the input from the microphones, resampled the files from a sample rate of 44,100 to a sample rate of 11,025, and saved them to files encoded with the identification number of the frog that was recorded, the experiment that was performed, and the date and time of the recording. The information was then extracted from the file name and imported to an SQL database.

These sound files were manually inspected using CoolEdit to determine whether the focal frog called, and to identify and isolate individual calls for each male. The individual calls from the male call recordings were then processed by Signal, which produced quantified spectral and temporal call characteristics for each call. These data were input to the SQL database. Output consisting of the average call characteristics of 30 calls per male was then used as input to the SPSS software for statistical analysis.

File management was an on-going task in this research project. Each file required manual inspection to ensure there were no problems with the playback and recordings; this is not a task that is automated easily or reliably. Because of the length of the evoked vocal response experiments, file sizes exceeded 100 megabytes per frog. These files were moved to external hard drives and archived on CD-ROMs for security. Using CDs also allowed several files to be analyzed simultaneously by several undergraduate research assistants.

The computer systems used in this project performed an invaluable role in maintaining the acoustic environments, recording data from behavioral experiments, and analyzing sound files. Researchers looking to use computers should invest in training to make maximum use of systems such as Linux and Matlab.

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